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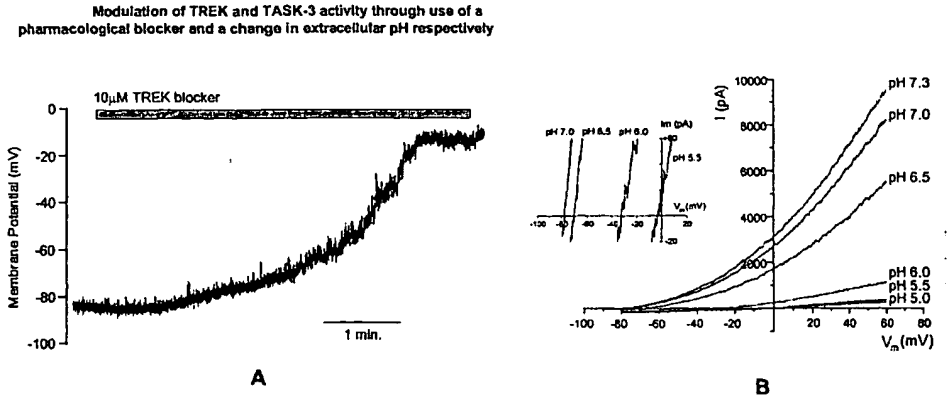

(57) Abstract: The present invention provides means for the skilled person to develop a high-throughput, plate-based voltage-dependence assay by transfecting a 'control' recombinant ion channel into a cell background which expresses the target ion channel. The term 'target ion channel' as used herein refers to the ion channel protein for which a modulator is sought. Development of a plate-based voltage-dependence assay will enable compounds to be ranked in terms of voltage-dependence at a very early stage in the drug discovery progression path. This invention provides the dual advantages of greatly increasing the chances of identifying voltage-dependent compounds, and of decreasing the requirement for electrophysiology. In addition, this method can be applied to help increase the signal-to-background for an ion channel assay, and to improve the ability of the user to generate stable cell lines expressing a target ion channel.

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Introduction

Ion channels are a proven target for drug discovery, and many ion channel modulators are currently in clinical use for the treatment of pain, epilepsy, hypertension and other disease states. A 'modulator' can be defined as any agent which alters the functional activity of an ion channel within a cell, and may represent a 'channel opener' (functional agonist), a 'channel blocker' (functional antagonist) or an agent that alters the level of expression of a channel protein at the cell membrane. An increase in expression will lead to greater numbers of ion channels, which will lead to an increase in overall activity. Conversely, a decrease in expression will lead to a smaller overall activity. The major challenge in development of ion channel modulators is to achieve selectivity for the target ion channel over other related ion channel subtypes, and for channels in the target tissue. As with other target classes, a degree of selectivity can be achieved through an iterative process of chemical synthesis around a pharmacophore of known activity, followed by bioassay. However, of equal importance in ion channel drug discovery has been the utility of three inter-related features of ion channel pharmacology: 'voltage-dependence', 'use-dependence' and 'frequency dependence', each of which arise as a result of an ion channel modulator binding preferentially to one or more conformational state of the channel protein.

Several definitions will be required to facilitate the ensuing description of voltage-dependence: Membrane potential is defined as the potential-difference or difference in voltage across the cell membrane, and is generated by a small imbalance in electrical charge between intracellular and extracellular compartments. Movement of positively charged ions (Na^+, K^+, Ca^{2+}) across the cell membrane, from cytoplasm to extracellular solution results in the interior of the cell becoming more negatively charged with respect to the exterior. This increase in polarity of charge across the plasma membrane is called hyperpolarisation. Conversely, movement of positively charged ions from extracellular solution to cytoplasm, results in a decrease in charge polarity and leads to a depolarisation of the cell. The reverse scenario occurs with anion flux,

with movement of negatively charged chloride ions from extracellular solution to cytoplasm resulting in hyperpolarisation, and movement from cytoplasm to extracellular solution generating a depolarisation. By convention, cellular membrane potentials are given a negative value, with hyperpolarisation of the cell giving a larger negative value, and depolarisation a smaller negative value. A depolarised membrane potential is generally referred to as a 'positive membrane potential', and a hyperpolarised potential as a 'negative potential'. Typical mammalian cell resting membrane potentials (i.e. membrane potential in the absence of dynamic changes in electrical activity such as action potentials) range from -90mV in cardiac ventricular myocytes to -20mV in the Chinese Hamster Ovary (CHO) cell line.

Ion channel proteins are known to exist in a number of different conformational states, referred to as gating states. While single-channel electrophysiological recordings have shown that ion channel gating can be extremely complex, for example involving multiple closed and open states (Hille, 1992), a simplified scheme is generally used for discussion purposes: Thus, for voltage-gated ion channels a channel can be viewed as residing in one of 3 gating states – closed (no ion permeation), open (ion flux occurs) and inactivated (no ion permeation; channel cannot be opened by depolarisation), although it should be noted that some channels do not exhibit an inactivated state. Transition between gating states is voltage-dependent and at any given time an equilibrium exists between these gating states, with the proportion of channels residing in each state depending upon the cellular membrane potential. For ligand-gated ion channels a similar three-state model is used, with closed (no ligand present; no ion permeation), open (ligand-bound; ion flux occurs) and desensitised (no permeation; channel cannot be opened by addition of ligand) gating states.

Many ion channel modulators have been shown to bind preferentially to a specific gating state or states. For example, the voltage-gated sodium channel blocker Lamotrigine is thought to bind to the open and inactivated states of the Brain II sodium channel protein (Xie et al.,1995; Kuo & Lu,1997). Preferential binding to a particular gating state may occur through an increase in channel affinity for the ion channel modulator, or simply through improved access of the drug to its binding site on the channel. As discussed above, at any given

membrane potential voltage-gated sodium channels will be distributed between the closed, open and inactivated states. Transition between gating states is dependent upon resting potential, and therefore the number of channels in the inactivated state will increase as the resting membrane potential moves closer to zero mV (i.e. during depolarisation). Therefore, for a sodium channel blocker that only binds to channels that are in the inactivated state, the proportion of drug-bound (and therefore blocked) channels increases with more positive membrane potentials, and in this way the drug shows voltage-dependent block.

Voltage-dependent block generally refers to a steady-state change in drug affinity as a result of a difference in resting membrane potential. There are several examples of voltage-dependent compounds that are used in the clinic. For example a number of dihydropyridine calcium channel antagonists, which have clinical utility for their effects on vascular smooth muscle show a marked voltage-dependence. Thus, nitrendipine which is thought to bind preferentially to the inactivated state of the L-type calcium channel, has a 2000-fold greater affinity at a holding potential of –15mV compared to that seen at a holding potential of –80mV (Bean,1984). Holding potential is defined as the membrane potential at which an electrophysiology assay is carried out, as controlled by the voltage-clamp amplifier. One can also consider the possibility of 'reverse voltage dependence', i.e. the situation whereby an ion channel blocker shows increased potency at more hyperpolarised membrane potentials. A theoretical example of this type of compound would be an open channel blocker of the hyperpolarisation activated potassium channel HCN1, which would be expected to increase in potency with membrane hyperpolarisation.

A related phenomenon to voltage-dependence of compound action is that of use-dependent block. Use-dependence is generally associated with more dynamic changes in membrane potential such as those occurring during a neuronal action potential, where membrane potential can transiently depolarise from -60mV to a peak value of at least 0mV. Returning to the example of Lamotrigine block of brain II sodium channels, it can be clearly demonstrated that the efficacy of Lamotrigine block is increased when a train of depolarising pulses is applied to the cell (Xie et al, 1995). This result can be reconciled if we make the assumption that lamotrigine only binds to the inactivated state of the

sodium channel. As discussed above, at rest a certain proportion of sodium channels will lie in the inactivated state and will bind Lamotrigine. However, when a train of depolarising pulses (or action potentials) is applied to the cell every sodium channel is driven through a gating cycle of closed-open-inactivated-closed and therefore becomes accessible to block by Lamotrigine. In this way, the use-dependent block by Lamotrigine can be far greater than 'tonic block' at any given drug concentration. Tonic block can be defined as the inhibition of ion channel activity recorded when a modulator is applied to the resting cell, in the absence of a voltage-clamp pulse train or a train of action potentials. Use-dependence of ion channel modulators can confer a high degree of selectivity for disease tissue over normal tissue and can therefore limit the plasma concentration required in the clinic. For example, Lamotrigine's success as an anti-convulsant is based upon its ability to discriminate between 'normal' brain tissue and hyperexcitable neuronal tissue, where action potential burst firing occurs and use-dependence of Lamotrigine comes into play (Xie et al.,1995). Use-dependent block has also been demonstrated in ligand-gated ion channels. For example, local anaesthetics are potent open-state blockers of the nicotinic acetylcholine receptor (Hille, 1992).

The terms use-dependent block and frequency-dependent block are often used interchangeably. However, there is a subtle distinction between the two processes and it is probably more accurate to refer to frequency-dependence as a sub-division of use-dependent block: Frequency-dependent block indicates that the efficacy of block by an ion channel modulator increases with increased frequency of channel gating. Use-dependent block indicates that efficacy of block increases as the channel spends an increased proportion of time away from the closed / resting gating state. This difference can be most clearly illustrated by considering the effects of an ion channel modulator during two different voltage-clamp protocols. In one protocol, a train of 2ms depolarising stimuli are applied from –90mV to 0mV every 1s. In a second voltage-clamp protocol, a change is made such that the voltage-clamp pulse width is increased from 2ms to 10ms. If an ion channel modulator is use-dependent then one would expect to see an increase in efficacy of block with increased pulse duration, as the proportion of time away from the resting state increases from 0.002 to 0.01. With this example, there is no change in frequency of stimulation

– 1Hz in each case – and therefore we have separated use-dependence of compound action from frequency dependence.

From the discussion above, it should be clear that voltage-dependence, use-dependence and frequency-dependence are highly inter-related phenomena, and in practice the majority of voltage-dependent ion channel modulators are also use- and frequency-dependent. Thus, if it were possible to develop a plate-based assay to identify voltage-dependent ion channel modulators, then this assay should also detect use- and frequency dependent compounds. For the remainder of the specification, the term voltage-dependence will be used as a global term incorporating all three mechanisms.

There is a further mechanism whereby a compound can show voltage-dependence. If a compound is charged, and if the drug binding site lies within the electrical field of the cell membrane, then the rate constants for drug binding and unbinding will be voltage-dependent (Woodhull, 1973). Thus, in this situation the potency of an ion channel modulator will be affected by membrane potential, and the compound will show voltage-dependence. Binding of Mg^{2+} ions and polyamines to (distinct) sites on the NMDA receptor shows voltage-dependence (Yamakura & Shimoji, 1999), and therefore an ion channel modulator which acted at either of these sites would be expected to show a similar property.

From the above examples it is clear that voltage- and use-dependence are key features for any ion channel modulator. Indeed in many drug-discovery projects voltage- / use-dependence may be viewed as a key feature of a lead molecule. The essential element in determining whether a modulator of an ion channel of interest (herein termed the target ion channel) is voltage-dependent lies in being able to compare the compound's activity against the target ion channel at two different membrane potentials (and therefore in two different gating configurations). This involves changing the membrane potential of the cells in which that target ion channel is expressed. To date, this has been achieved by electrophysiology using the voltage-clamp technique (either whole-cell patch-clamp in mammalian cells, or two-microelectrode voltage-clamp in *Xenopus* oocytes or multicellular preparations) which allows the user to have exquisite control of cellular membrane potential. The same techniques can be used to

study use-dependence, with the user applying a train of voltage-clamp pulses and comparing tonic and use-dependent block. However, electrophysiology has two well-documented draw-backs: a requirement for a highly skilled worker to carry out the experiments; and a very low throughput (typically 10-25 compounds per week). In practice, the latter problem has resulted in a very small number of compounds being tested for voltage- / use-dependence in a typical drug discovery program, with these features having to be acquired largely through serendipity or through extensive SAR chemistry. In ion channel research current plate-based assay methodologies include voltage-sensitive dyes (oxonol dyes - e.g. DiBAC(4)$_3$ (Terstappen et al., 2001); Aurora FRET probes (England, 1999)), ion sensitive dyes (e.g. Fluo-4 for calcium; Thomas et al.,2000) or ion flux measurements. However, in each of these assay formats it is only possible to detect tonic actions of an ion channel modulator, and to date no plate-based ion channel assay has been configured to detect voltage-dependence. Thus, there remains a need for high throughput ion channel assays to screen for voltage dependent compounds.

The present invention provides means for the skilled person to develop a high-throughput, plate-based voltage-dependence assay by transfecting a 'control' recombinant ion channel into a cell background which expresses the target ion channel. The term 'target ion channel' as used herein refers to the ion channel protein for which a modulator is sought. Development of a plate-based voltage-dependence assay will enable compounds to be ranked in terms of voltage-dependence at a very early stage in the drug discovery progression path. This invention provides the dual advantages of greatly increasing the chances of identifying voltage-dependent compounds, and of decreasing the requirement for electrophysiology. In addition, this method can be applied to help increase the signal-to-background for an ion channel assay, and to improve the ability of the user to generate stable cell lines expressing a target ion channel.

Summary of the Invention

The present inventors accordingly provide a stable cell line which expresses a target ion channel of interest, characterised in that the cell line also comprises a further, control ion channel which can be utilised to set the resting membrane potential to a desired level. The invention further provides a screen which comprises such a cell line, preferably the screen is for voltage dependent substances.

In another aspect of the invention is provided a high throughput method for the identification of a voltage dependent substance that modulates ion channel activity and/or expression, which method comprises:

(i) contacting a test substance and said ion channel, and

(ii) determining the effect of the test substance on the activity and/or expression of the said ion channel, thereby to determine whether the test substance modulates ion channel activity and/or expression and

(iii) comparing the effect of said test substance on the activity and/or expression of the said ion channel in cell lines with varying membrane potential.

Preferably the test substance is contacted with an ion channel expressed in a cell line and the effect of the test substance is compared with the effect of the same test substance on the same ion channel in the same cell line at a different membrane potential.

In another embodiment of the invention is provided a method of altering the resting membrane potential of cells in a plate based assay format comprising expressing in said cells a control ion channel, preferably a potassium ion channel.

The invention further provides a high throughput method of screening for voltage dependent compounds comprising the steps of contacting a ligand with its respective ion channel in the presence or absence of a test compound and comparing the activation of said channel in the presence or absence of said compound, said method characterised in that the membrane potential at which the assay is conducted has been set to a pre-determined level.

Further provided is a plate based high throughput screen comprising a stable cell line engineered to express on its surface a target ion channel of interest, characterised in that the resting membrane potential of said stable cell line has been modulated by the expression of a cloned ion channel, preferably a potassium ion channel.

Detailed description of the Invention.

Throughout the present specification and the accompanying claims the words "comprise" and "include" and variations such as "comprises", "comprising", "includes" and "including" are to be interpreted inclusively. That is, these words are intended to convey the possible inclusion of other elements or integers not specifically recited, where the context allows.

The present inventors have demonstrated methods of re-setting the resting membrane potential of a cell, and have importantly determined that voltage-dependent compounds can be identified in a high throughput, plate-based assay format by controlling the resting membrane potential of the cells which express the target ion channel of interest within the assay. A preferred method of setting resting membrane potential comprises co-expression of a recombinant 'control' ion channel, which is preferably a potassium ion channel. However, other types of control ion channel (endogenous or recombinant) can also be considered, as can ionophores such as valinomycin. As used herein, the term "control ion channel" should be interpreted accordingly. The inventors also demonstrate that methods of controlling resting membrane potential can have a major utility in the development of plate-based assays for a target ion channel, either through rescuing voltage-gated target ion channels from the inactivated state or through improvements in assay signal-to-background.

The resting membrane potential of a cell is primarily determined by the sum of the individual ionic concentration gradients for Na^+, K^+, Ca^{2+} and Cl^- across the cell membrane, and hence it may be controlled by alteration of the gradient for any one of these ions. This may be achieved in a variety of ways, for example by modulating the activity of the plasma membrane Na^+/K^+ ATPase (sodium pump),

by modulating endogenous potassium channels that control potassium efflux, or through the use of ionophores such as monensin or gramidicin to increase sodium influx, or valinomycin to increase potassium efflux. Preferably, however, the resting membrane potential is controlled by the introduction of a cloned ion channel.

Where the membrane potential is altered by the introduction of a cloned ion channel, this ion channel can be utilised to control the resting membrane potential of the cell. The control channel may be a channel that is selective for Na^{2+}, K^{+} Ca^{2+} or Cl^{-} ions, or a non-selective ion channel, but is preferably a potassium channel. Potassium-selective ion channels are preferred as control ion channels, as the membrane potential of most host mammalian cell-lines is relatively positive compared to the normal potassium equilibrium potential, and therefore large shifts in resting membrane potential can be achieved through expression or activation of the control channel. The potassium equilibrium potential is defined as the membrane potential at which there is no net flux of potassium ions across the cell membrane, and can be determined using the Nernst equation. Under typical conditions, with 145mM intracellular K^{+} and 5mM extracellular potassium, the potassium equilibrium potential lies at approximately -85mV.

The resting membrane potential of a cell is determined by the sum of the various steady-state ionic conductances present in the plasma membrane as defined by the Goldman Hodgkin Katz equation (Hille,1992). The influence of a particular ion channel on the membrane potential is determined by the transmembrane concentration gradient for the permeating ion, and the ionic permeability of the cell membrane. Thus, introduction of a control potassium channel increases the permeabililty of the cell membrane to potassium ions, allows positively charged K^{+} ions to flow across the cell membrane from cytoplasm to extracellular solution, and hyperpolarises the cell's resting membrane potential towards the potassium equilibrium potential.

The driving force for potassium flux through the control ion channel is proportional to the difference between the cell membrane potential and the equilibrium potential for the permeating ion (driving force = $E_m - E_{ion}$), therefore

expression/activation of a control potassium channel will have a greater effect when the resting membrane potential of the host cell is relatively depolarised. Commonly used host cell lines such as HEK293 and CHOK1 typically have a resting membrane potential of between –10mV and –45mV, and are therefore preferably used for manipulation by co-expression of a control potassium channel and a target ion channel.

In more general terms it can be stated that functional expression of any control ion channel or ionophore will tend to drag membrane potential towards the equilibrium potential for the permeating ion. It will be apparent to a person skilled in the art that the use of various types of control ion channel could also be used to reset the resting membrane potential. The effects of control channel expression/activation on resting membrane potential will depend critically upon the Nernst equilibrium potential for the permeating ion. The control ion channel may also be a sodium ion channel, for example a non-inactivating sodium channel such as the amiloride-sensitive epithelial sodium channel (ENaC) which would be expected to depolarise the resting membrane potential towards the sodium equilibrium potential (approximately +60mV under physiological conditions). Alternatively, the control ion channel may be a chloride-selective control channel or a calcium-selective control cell, which would reset the resting membrane potential towards the Nernst equilibrium potential for chloride and calcium ions respectively.

Plate-based assays can be developed to screen compounds against a stable cell line co-expressing the target ion channel of interest, plus a control ion channel . Such stable cell lines include higher eukaryotic cell lines such as mammalian cells or insect cells, lower eukaryotic cells such as yeast, or prokaryotic cells such as bacterial cells. For example, the cell line used may be Human Embryonic Kidney (HEK293T), Chinese Hamster Ovary (CHO), HeLa, BHK, 3T3 or COS. A person skilled in the art will be aware of how to prepare stable cell lines expressing a protein of interest. Guidance may be found, for example, in Sambrook et al, Molecular Cloning: a Laboratory Manual. 2nd Edition. CSH Laboratory Press. (1989) the disclosure of which is incorporated herein in its entirety by reference. The cell line selected should be one which retains stable expression of control and target channels over cell passage, and

also allows for mature glycosylation and cell surface expression of both target and control ion channels. High transfection-efficiency transient transfection systems may also be used to express the control and target channels, for example the 'BacMam' baculovirus expression system (Kost et al.,2000). On some occasions, it may be appropriate to utilise a target channel stable cell line where an endogenous control channel is present. In this case stable expression of target ion channel only will be required.

The target ion channel is the ion channel for which voltage dependent modulators are sought, or alternatively the ion channel in which a problematic plate-based assay is to be configured. When developing a voltage-dependence assay the target channel is preferably an ion channel which has different gating states, in which transition between gating states is dependent upon the cellular membrane potential. Alternatively, the target channel may be a channel in which modulators bind to a site within the membrane electrical field. The target ion channel may be a voltage-gated ion channel such as a potassium, sodium, calcium or chloride channel, a ligand-gated channel such as the NMDA receptor, or a constitutively active channel.

Potassium channels are particularly appropriate for use as a control ion channel if they are open at negative resting potentials (between –50mV and –90mV), are non-inactivating over a range of membrane potentials, and have known pharmacological tools that can act as channel openers and blockers providing additional control over the channel, and therefore of resting membrane potential. There are many types of potassium channels that can be used as a control channel, each providing different set of biophysical parameters and having different pharmacological tools. It is well known that a functional potassium channel comprises a tetramer of 4 alpha (α) sub-units with additional beta sub-units also present (e.g. minK for the KCNQ1 channel (Sanguinetti et al.,1996), or Kvβ sub-units for other voltage-gated potassium channels (Nakahira et al.,1996). Where the tetramer comprises 4 identical sub-units, the channel is referred to as a homomeric potassium channel, and where the tetramer comprises several different channel sub-units the channel is referred to as a heteromeric potassium channel. The present invention includes the use of all combinations of potassium channel sub-units as control ion channels. Thus, in each example detailed below

the control channel may comprise a homotetramer or heterotetramer of sub-units from the same channel family, and may be expressed either with or without auxilliary beta sub-units.

Preferably, if the control ion channel is a potassium channel, it is a KCNQ potassium channel, such as a KCNQ2/3 heteromeric channel; a Calcium-activated potassium channel, such as a BK, SK1, SK2, SK3, or SK4/IKchannel; an Inward rectifier potassium channel, such as the GIRK family of channels, the Kir family, or a K_{ATP} potassium channel; a two-pore potassium channel, such as TREK, TASK or TRAAK or a member of the eag or erg family of potassium channels such as . hERG.

Expression of a control ion channel in the target channel stable cell line provides a basis for regulation of resting membrane potential. However, it is possible to further regulate the cell line membrane potential. A standard equation widely used in electrophysiology, is that whole-cell ionic current (I) is equal to the product of the number of channels expressed at the plasma membrane (N), the single channel current (i) and the probability of a channel being open at a given time (open channel probability; P_o):

Thus, $I = N \times i \times P_o$

The same 3 factors are also key when considering the whole-cell ionic conductance for a particular ion, and the influence that this conductance has upon the resting membrane potential. From this, it is clear that the activity of a control channel and hence the membrane potential of the cells expressing it can be regulated in 3 ways:

1) By modulating the number of control channels.
   This can be achieved by increasing the total cellular expression level of the control channel protein, or by increasing the proportion of expressed control channels that are functional at the cell surface. Total protein expression level can be regulated through choice of expression vector (e.g. use of vectors with different promoters, inducible-expression vectors, baculovirus expression systems) or chemically through the use of sodium butyrate (Nash et al.,2001).

For some channel types, an increase in the number of control channels at the cell surface can be achieved by co-expression of an additional sub-unit or of a trafficking factor. For example, co-expression of KCNQ2 and KCNQ3 has been shown to increase the number of functional channels at the surface membrane (Schwake et al., 2000; JBC 275 13343-8).

2) By modulating the single channel current

Single channel current amplitude is determined by the biophysical properties of a channel and by the driving force for ion flux through that channel. In turn, driving force is proportional to the difference between the cell's membrane potential and the equilibrium potential for the permeating ion (driving force = $E_m - E_{ion}$). Thus, the single channel current amplitude (and therefore the influence of the control channel on membrane potential) can be altered by changing the extracellular concentration of the permeating ion for the control ion channel, and therefore re-setting the equilibrium potential. For example, where the control ion channel is a potassium channel, the extracellular concentration of potassium can be varied. Where it is a sodium, chloride or calcium channel, the extracellular concentration of sodium, chloride or calcium respectively can be varied.

3) By modulating the probability of channel opening

Probability of channel opening can be modulated pharmacologically, or through exploitation of the physiological gating mechanisms of the channel protein. For example, the probability of opening of a calcium-activated potassium channel (e.g. IK) will be increased by any experimental manoeuvre that increases the levels of intracellular calcium. Pharmacological tools have been identified for most ion channel proteins, and either a channel blocker or opener may be utilised depending upon the identity and biophysical properties of the control ion channel.

The present invention has many applications that will be apparent to a person skilled in the art. These will now be described in further detail:

1) Voltage-dependence assay

From the various experimental paradigms detailed herein, it is clear that in this fashion a compound can easily and rapidly be tested against the target ion channel at several different membrane potentials, for example two or more, such as three, four or five different membrane potentials. A comparison of the effect of compounds against the target ion channel at the different membrane potentials set in the assay enables determination of whether the compound is voltage dependent. The present invention allows this to be done in a high throughput or plate-based fashion, which has not previously been possible.

2) Improving signal-to-background

In another aspect of the invention, the control ion channel is used to improve assay results by increasing the signal to background ratio. This is a measure of the target ion channel signal amplitude compared to background 'noise' of the assay. The larger the ratio, the easier it is to accurately measure signals, and the more confidence one may have in the results. Improvements in signal-to-background may occur in several ways in the stable cell line of the invention. With some target ion channels, for example the cardiac sodium channel or T-type calcium channel, a large percentage of channels would be expected to lie in the inactivated state when expressed in host cell lines such as CHO and HEK293T that have a relatively depolarised resting membrane potential. This means that during an assay, one can be restricted to recording results from the sub-set of the channel population which do not lie in the inactivated state. Co-expression of a control potassium ion channel will hyperpolarise the cell, shifting channels out of the inactivated state to a closed gating state from which they can be activated. In this way, target channel signal amplitude, and therefore the signal-to-background ratio is increased.

The signal to background ratio may also be improved by increasing the driving force for ion movement across the cell membrane. This ionic driving force can be defined as $E_m - E_{ion}$, where E_m is the membrane potential and E_{ion} is the Nernst equilibrium potential for the target channel permeating ion. Under assay conditions where the concentration of the permeating ion is fixed on either side of the membrane, E_{ion} does not change. However, as the present inventors have demonstrated, cellular resting potential (E_m) can be modulated by functional expression of, and ionic flux through, a control ion channel. In the case of a

voltage-gated sodium or calcium channel as the target ion channel, where E_{ion} is positive (typically +50 to +70mV), hyperpolarisation of the resting potential using a control potassium channel will lead to a larger driving force for target ion flux, and an increase in amplitude of the target ion channel response. Conversely, in the case of a potassium channel as the target channel, E_{ion} is negative (typically around -85mV), and depolarisation of the resting potential using a control sodium channel will lead to a larger driving force for target ion flux, and an increase in signal amplitude.

3) Cell-line development

In another aspect, the control ion channel is used to aid development of the stable cell line. A number of target ion channels, including (but not restricted to) voltage gated calcium channels, are known to have detrimental effects on the cells expressing them. For example, if a voltage-gated calcium channel is the target channel, if a large proportion of the calcium channels lie in the open gating state, then there may be sustained calcium influx through these channels, causing calcium overload and cell death. Stable cell line construction can therefore be difficult, particularly if the cell line has a fairly positive resting potential such that more target channels lie in the open gating state, for example a CHO cell line. This is a particular problem where a non-inactivating 'window current' exists, such that a sustained calcium influx takes place over a range of membrane potentials. A so called 'window current' is seen for some voltage-gated channels, and occurs under specific biophysical conditions where ion channels are activated over a range of membrane potentials in which steady-state inactivation is less than 100%. A control ion channel can be utilised to ensure that the resting membrane potential is hyperpolarised, thereby shifting more of the target ion channels into the closed state, and reducing ion influx and subsequent cell damage. This aspect is particularly useful when screening for modulators of such a 'toxic' target ion channel, such as a voltage-gated calcium channel, which otherwise would present difficulties in terms of stable cell line development and cell line maintenance.

Screening methods – voltage-dependence assay

An important aspect of the invention is the realisation that by controlling the resting membrane potential it is possible to carry out high throughput screens to identify voltage-dependent substances. Drug discovery is dependent on the ability to screen many thousands of compounds simultaneously, and the present invention makes this possible.

The screens and screening methods of the present invention may be used to identify agents that bind to the target ion channel, agonists or antagonists which may modulate its activity, inhibitors or activators of the target ion channel activity, or agents which up-regulate or down-regulate expression of the target ion channel. In particular, the present invention is useful for identifying agents that have different effects at different resting membrane potentials.

Any suitable format may be used for the assay. In general terms, such screening methods may involve contacting a test substance with the target ion channel (which may be endogenously or recombinantly expressed), and determining the effect of the test substance on the activity and/or expression of the target ion channel. The assay should be done in cell lines with varying membrane potentials. As described herein, the membrane potential may be varied by any means, but is preferably by the expression of a control ion channel, and particularly preferably by the expression of a control potassium ion channel. Further variations in the membrane potential of the cells in the assay may be achieved if desired by use of a blocker or opener of the control ion channel, or alternatively by varying the extracellular concentration of the permeating ion for the control ion channel, and hence shifting the equilibrium potential for that ion. Preferably, control experiments should be carried out to demonstrate that the control channel opener/blocker has no direct effect upon the target channel. Similarly, any voltage-dependent modulators identified in the target ion channel assay should be tested for activity against the control ion channel in isolation.

When screening for voltage dependent compounds, the assay for modulators of the target ion channel is carried out using cells at two or more different resting membrane potentials. The detailed methods used to establish cell lines with two different resting potentials will depend upon the biophysical parameters of the control channel: However, these methods can be split into three basic groups

depending upon the type of control channel used. In all cases, determination of a compound's voltage dependence is carried out by a comparison of the potency of the compound at the various membrane potentials used in the assay.

i) Where a control channel shows constitutive activity, and this activity can be modulated pharmacologically (for example the KCNQ2/3 and TREK potassium channels, and the epithelial sodium channel):

In one aspect, an assay is designed such that some of the cells against which the compound is tested to do not comprise the control ion channel, and so have their natural resting membrane potential. Some of the cells against which the compound is tested do comprise the control ion channel, and hence the membrane potential of these latter cells is altered, depending on the control channel used.

In a second aspect, all cells against which the compound is tested comprise both the control and target ion channels, and some cells are assayed with pre-incubation of a pharmacological opener of the control ion channel, and some cells are assayed without preincubation with a pharmacological opener of the control ion channel.

In a third aspect, all cells against which the compound is tested comprise both the control and target ion channels, and some cells are assayed with and some cells without pre-incubation of a pharmacological blocker of the control ion channel.

In a fourth aspect, all cells against which the compound is tested comprise both the control and target ion channels, and cells are assayed following pre-incubation in buffer solutions of varying concentrations of the permeating ion for the control ion channel. The membrane potential of a cell varies depending on the concentration of the control ion in the buffer solution.

ii) Where the control channel does not show constitutive activity, but can be opened pharmacologically (for example the IK potassium channel; K_{ATP})

In one aspect, all cells against which the compound is tested comprise both the control and target ion channels, and some cells are assayed with and some without pre-incubation of a pharmacological opener of the control ion channel.

In a second aspect, all cells against which the compound is tested comprise both the control and target ion channels, and all cells are assayed following pre-incubation in a channel opener. Resting membrane potential can then be controlled by incubation in buffer solutions of varying concentrations of the permeating ion for the control ion channel to modulate ionic flux through the control ion channel.

iii) Where the membrane potential is set by using a selective ionophore, for example valinomycin for potassium ions, ionomycin for calcium ions and gramicidin which has dual selectivity for sodium and potassium ions.

In one aspect, an assay is designed such that some of the cells against which the compound is tested are pre-incubated with ionophore (e.g. valinomycin) and will therefore have a membrane potential which approximates to the potassium equilibrium potential, whereas other cells are not and so have their natural resting membrane potential.

In a second aspect, all cells against which the compound is tested are pre-incubated with an ionophore, and buffer solutions of various potassium concentration are used to modulate potassium flux through the ionophore, and to reset membrane potential to the new potassium equilibrium potential.

A person skilled in the art will appreciate that any combination of the above aspects may be used in order to lead to a wider variety of membrane potentials.

In all of the above cases, the preferred target channel assay format is a cell-based assay, which may constitute a whole-cell ligand binding assay, or a functional cellular assay in which membrane potential, ion flux, intracellular ion concentration or extracellular ion concentration is the read-out. Preferably the assay is carried out in a single well of a microtitre plate, although other formats

may be applicable. Assay formats which allow high throughput screening are preferred.

In the preferred format for identifying voltage-dependent, target channel modulators, a cell-based assay will be established in which activation of the target ion channel can be detected by means of a change in membrane potential, ion flux, intracellular ion concentration or extracellular ion concentration. This can be achieved at present using the following assay technologies: **membrane potential** - fluorescent, voltage-sensitive (potentiometric) dyes (e.g. DiBAC$(4)_3$) in conjunction with a Fluorometric Imaging Plate Reader (FLIPR) or other suitable assay platform; FRET/BRET-based membrane potential sensitive dyes in conjunction with the Aurora Biosciences VIPR or another suitable assay platform. **Ion flux** – rubidium flux assay for potassium channels; lithium flux assay for sodium channels; radioactivity or atomic force read-out used in these assays. **Ion concentration** – ion-sensitive fluorescent probes (e.g. Fluo-4 for calcium); aequorin assay for measurement of intracellular calcium.

Activation of the target ion channel may be achieved through addition of its endogenous ligand, addition of a known channel opener, depolarisation of the cell membrane (by addition of a high potassium solution, by electrical field stimulation, or indirectly through activation of a second control ion channel such as a P2X channel), addition of a buffer solution with a modified pH, addition of a buffer solution with a modified osmolarity, or activation through triggering a rise in intracellular calcium or sodium concentration.

In a plate-based assay for voltage-dependent compounds, a concentration-response curve will be constructed for a ligand against the target ion channel at two or more different resting membrane potentials. The choice of resting membrane potentials will be dictated by the particular biophysical parameters of the target ion channel. Depending upon the type of control ion channel or ionophore used to set the resting membrane potential, one or more different cell lines may be used in the assay. On completion of the assay, the potency of a ligand against the target ion channel will be compared at the various membrane

potentials. If the ligand is voltage-dependent, then one would expect to see a shift in potency.

Assays may also be carried out to identify substances which modulate target ion channel expression, for example substances which up- or down- regulate expression. Such assays may be carried out for example by using antibodies for the target channel to monitor levels of target ion channel expression, or through use of a ligand-binding assay. Preferably, this assay will be attempting to identify substances that have different effects at different membrane potentials.

The screens and screening methods of the present invention are amenable to the development of a high throughput assay. This means that a large number of compounds can be assayed simultaneously, at a membrane potential that is dictated by the experimenter. Preferably, at least 4000 data points per day will be generated in this assay format. This may be achieved by culturing the cells to be used in the assay in a microtitre plate, for example a 24 well, 96 well, 384 well or 1536 well plate, with preferably at least 40 plates assayed per experimental day.

Assays to compare the effects of compounds at different membrane potentials may be carried out in several ways, for example one entire microtitre plate may contain cells at one membrane potential, whilst a separate microtitre plate contains cells at another membrane potential, and the results from each plate are compared. Alternatively, the same plate may comprise cells at different membrane potentials, and the results from each well are compared. This is largely a matter of personal preference and assay design, and all such variations are included within the scope of the invention.

The present inventors' ability to preset the resting membrane potential in a high throughput system also provides a method of improving a plate based assay for ion channel activation, which method is characterised in that the membrane potential has been set to a pre-determined level. By improving is meant, for example, the ability to screen for voltage dependent compounds, the ability to screen against target ion channels that had previously been difficult to develop an assay for, the ability to increase the signal to noise ratio for a target channel

assay, and the ability to generate and maintain a stable cell line expressing the target ion channel of interest, all as described herein.

The invention will now be described further, by way of the following non-limiting examples.

Example 1. Constitutively active control potassium channels:
i) Utility of a KCNQ potassium channel as the control channel

The resting membrane potential of a cell can be hyperpolarised by expression of a KCNQ potassium channel. Figure 1a (filled circles) shows the mean current-voltage relationship for the heteromeric KCNQ2/KCNQ3 potassium channel expressed in Xenopus oocytes. KCNQ channels are voltage-gated potassium channels and the activation threshold for the KCNQ2/3 channel in oocytes is approximately –60mV. An important feature of the KCNQ2/3 channel is that the channel does not exhibit time-dependent inactivation (see inset to Figure 1a), but rather moves slowly between closed and open gating states in response to a change in membrane potential. The result of this combination of biophysical parameters is that any cell expressing the KCNQ2/3 channel will have a steady state KCNQ-mediated potassium conductance at all membrane potentials above the threshold for KCNQ channel activation. In recombinant expression systems, where KCNQ channels are over-expressed relative to other endogenous conductances, this means that the resting membrane potential will be re-set to the KCNQ channel activation threshold. Small depolarisations from this membrane potential will lead to activation of the KCNQ2/3 conductance, and this conductance will dampen cellular excitability and effectively put a 'brake' on any change in resting potential. The effects of KCNQ2/3 channel expression on membrane potential are illustrated in Figure 1b, where membrane potentials measurements from a Xenopus oocyte are shown. In these experiments 'normal' resting membrane potential in un-transfected oocytes was between –25 and –30mV. Injection of cRNA encoding the KCNQ2 and KCNQ3 channels led to a shift in resting membrane potential to approximately –65mV.

Retigabine has recently been identified as an opener of KCNQ2/3 potassium channels (Main et al, 2000). As shown in Figure 1a (open circles), retigabine acts by shifting the voltage-dependence of KCNQ channel activation such that KCNQ currents are recorded at all membrane potentials positive to –80mV. The effects of this shift in activation threshold are shown in Figure 1b. Retigabine application to an oocyte expressing the KCNQ2/3 heteromeric channel led to a concentration dependent hyperpolarisation.

Figure 2 shows data from a FLIPR/DiBAC plate-based assay. In the experiment shown in Figure 2a, CHO KCNQ2/3 cells were pre-incubated with various concentrations of the KCNQ opener retigabine, and basal DiBAC signal is plotted against retigabine concentration. Note that a low number of DiBAC counts reports a relatively hyperpolarised membrane potential, whereas a higher number of DiBAC counts is indicative of a more depolarized resting membrane potential. In agreement with the oocyte electrophysiology data shown in Figure 1b, the CHO KCNQ2/3 cell line clearly has a lower basal DiBAC count that that seen in wild type (wt) CHO cells, suggesting that the resting membrane potential is hyperpolarised following expression of the KCNQ2/3 channel. Pre-incubation with retigabine produced an additional concentration-dependent decrease in DiBAC signal, which again is consistent with the hyperpolarisation seen in oocytes following retigabine application. Further experiments were carried out using the KCNQ channel blocker XE991 (Wang et al.,1998). As shown in Figure 2b, pre-incubation in XE991 led to an increase in DiBAC counts, which is consistent with depolarization of the CHO cell membrane.

From the experiments outlined above it is clear that the KCNQ2/3 potassium channel can be used as a control potassium channel to reset the membrane potential. Other members of the KCNQ channel family show similar biophysical and pharmacological characteristics, and will therefore be equally compatible. Comparison of cells at different resting membrane potentials can be achieved by looking at 'wild type' CHO cells versus CHO KCNQ2/3 cells, or alternatively by pharmacological modulation of membrane potential in the CHO KCNQ2/3 cell line using KCNQ channel openers or blockers.

Example 2. Constitutively active control potassium channels:
ii) Utility of a two-pore potassium channel as the control channel

The resting membrane potential of a cell can be substantially altered by expression of a two pore domain (2P) background potassium channel such as TREK-1, TRAAK or TASK-3. Figure 3a shows the mean current-voltage relationship for HEK293 cells transiently transfected with cDNA encoding the TRAAK channel. A current-voltage curve is also shown for HEK cells transfected with cDNA for the transfection marker, green fluorescent protein (GFP), which can be considered as a negative control in this experiment. In GFP expressing cells, a linear current-voltage curve is seen which reverses (i.e. reverses polarity from an inward to outward current at the reversal potential) at approximately –20mV. In HEK cells transfected with TRAAK cDNA much larger currents were recorded, indicative of potassium flux through the constitutively active TRAAK channel. The TRAAK current-voltage relationship is outwardly rectifying (i.e. conductance is greater when current flows in the outward direction) and has a reversal potential of approximately –80mV. The reversal potential or 'zero current' reading in a patch clamp experiment is indicative of the resting membrane potential of the cell. Thus, when the 'command potential' dictated by the experimenter is equal to the resting membrane potential of the cell, no current injection by the patch clamp amplifier is required to 'voltage-clamp' the cell membrane. If the zero current value is taken as a measure of resting membrane potential, then it can be surmised that expression of TRAAK hyperpolarises the resting membrane potential by approximately 60mV, from –20mV to –80mV. This shift in zero current potential is shown more clearly in the inset to Figure 3a.

Figure 3b shows the results of an very similar experiment in which TASK-3 is used as the control potassium channel. Note the shift in zero current potential from approximately –25mV to –65mV following expression of the constitutively active TASK-3 channel. With both TRAAK and TASK-3 channels, functional expression confers a steady-state potassium conductance to the cell membrane, and will tend to drag the resting membrane potential (and therefore zero current potential) towards the potassium equilibrium potential (E_K). Under the current recording conditions, with 5mM extracellular potassium and 140mM intracellular

potassium, E_K is predicted to lie at approximately –85mV. Under these recording conditions, the magnitude of the shift in resting membrane potential recorded following expression of the two-pore potassium channel will depend upon the biophysical parameters of the channel used (i.e. single channel current, probability of channel opening), the expression level (i.e. number of functional channels) and also the contribution of other endogenous ionic conductances to the cellular resting membrane potential.

As described above, expression of two-pore potassium channels will hyperpolarise the cellular resting membrane potential towards the potassium equilibrium potential. In order to assay cells at different membrane potentials, two approaches can be considered: First, an assay can be set up whereby target ion channel responses are compared in 'wild type' untransfected host cells, and these results can then be compared to those seen in cells expressing the two-pore potassium channel.

The second method which can be used is to work with a single cell line expressing a two-pore potassium channel of choice, and then to modulate the activity of this channel such that resting membrane potential is reset. Figure 4 demonstrates two methods whereby modulation of two-pore potassium channel activity re-sets the resting membrane potential.

Figure 4a shows a membrane potential recording from a HEK293 cell expressing the TREK two-pore potassium channel. As with TRAAK and TASK-3, expression of the TREK channel hyperpolarises the resting membrane potential towards the potassium equilibrium potential; in this cell the resting membrane potential is –85mV. Addition of a TREK channel blocker leads to a slow depolarization to a steady-state value of approximately –15mV, which approximates to the resting membrane potential of untransfected HEK293 cells. Thus, a voltage-dependence assay could be configured by comparing TREK expressing cells with/or without pre-incubation in the presence of TREK blocker. It may also be possible to generate cells with a range of membrane potentials simply by pre-incubating in various concentrations of a TREK-1 blocker.

Figure 4b demonstrates a second method whereby modulation of two-pore channel activity can be used to set the cellular resting membrane potential. This method takes advantage of the observation that changes in extracellular pH modulate activity of the TASK-3 two-pore potassium channel (Meadows & Randall,2001). Current-voltage curves are shown for a voltage-clamped HEK293 cell expressing TASK-3. Whole-cell currents were sampled by applying a voltage-ramp from –80 to +80mV. As shown in the main panel of Figure 4b, acidification of the extracellular recording solution leads to a pH-dependent block of the TASK-3 channel. This block is associated with a concomitant shift in the zero-current potential (see inset) from –78mV at pH 7.0 to –4mV at pH 5.5. Thus, in a similar manner to that described above for TREK-1 and a pharmacological blocker, a voltage-dependence assay could be configured by assaying target channel activity at a range of extracellular pH's, and therefore with a range of TASK-3 channel activities and resting membrane potentials. Such an assay would be configured using a single cell line, and would not require comparison to the 'wild type' host HEK293 cell response.

Example 3. Use of a control potassium channel that shows a low level of constitutive activity when expressed at moderate levels, but has a profound effect upon membrane potential following over-expression: Utility of an ATP-sensitive potassium channel (SUR1 plus Kir 6.2) as the control potassium channel.

The ATP-sensitive ion channel (K_{ATP}) comprises two sub-units: an inward-rectifier potassium channel (Kir6.2) forms the channel pore, and is associated with a second protein, the sulfonylurea receptor (SUR1 or SUR2) that dictates the pharmacological properties of the channel. Under normal physiological conditions the K_{ATP} channel is closed, however in conditions of metabolic demand the channel opens in response to a drop in intracellular ATP concentration. Pharmacological openers and blockers of the K_{ATP} channel have been identified, with diazoxide (opener) and glyburide (blocker) acting as selective tools for channels containing the SUR1 sub-unit.

Figure 5a shows data from a FLIPR/DiBAC assay in which levels of functional K_{ATP} channel expression have been titrated using a recombinant bacculovirus expression system ('BacMam'). CHO cells that had been transduced with varying amounts of Kir6.2/SUR-1 viral stock, and had been shown electrophysiologically to express functional K_{ATP} channels, were pre-incubated in 100 μM diazoxide to open the channels. 20 μM glyburide was then added 'on-line' to the cells during the FLIPR assay to reverse the diazoxide-activated DiBAC response. In Figure 5a, the amplitude of the DiBAC response to glyburide addition is plotted against BacMam viral load. In the absence of virus no response to glyburide was seen. However, with increasing viral load, a virus concentration-dependent increase in glyburide response was recorded, suggesting that there is an increase in the number of diazoxide-activated functional K_{ATP} channels in the pre-incubation period prior to glyburide addition, and a concomitant hyperpolarisation of the cellular membrane potential. These conclusions are supported by data from 'perforated patch' electrophysiological recordings (data not shown) where a diazoxide-activated hyperpolarisation from -41.4 ± 3.8mV to –65.3 ± 4.1mV is seen in cells transduced with Kir6.2/SUR-1 viral stock (in native cells from this batch of CHO cells a typical value for resting membrane potential is –30 to -45mV).

Figure 5b shows the effects of K_{ATP} channel overexpression on resting membrane potential (as measured using DiBAC). In this experiment CHO cells were transduced with concentrations of virus that were 10-fold higher that those used in the experiment described above. Under these conditions of over-expression, the K_{ATP} channel appears to dominate the cellular membrane potential without a requirement for pharmacological opening of the channel. Thus, glyburide responses were measured in the absence of diazoxide in CHO cells infected at concentrations greater than 0.04×10^7 virus, suggesting that a significant K_{ATP} channel-induced hyperpolarisation is occurring at these high levels of expression. These data suggest that each individual K_{ATP} channel does show a low level of functional activity in the absence of diazoxide, but that this activity only has a significant effect upon the cellular membrane potential at very high levels of channel expression.

The data shown in Figure 5 illustrate two important facts: First, the K_{ATP} channel (Kir6.2 + SUR1) can be used as a control potassium channel to regulate cellular resting membrane potential. Depending upon expression level, the K_{ATP} channel can function as a constitutively active control channel in a similar manner to TRAAK (see example 2 above), or as a non-constitutive control channel in the manner of the IK potassium channel (see example 4). The second important point is the demonstration that the effects of a control channel on membrane potential can be regulated by changing the number of functional channels expressed at the cell surface ('N' in the equation I = N.i.Po). Bacculoviral expression of a control channel may provide a means of titrating the resting membrane potential of a cell to that required in a plate-based assay, and will represent a significant time-saving when compared to construction of stable cell lines using conventional methods.

Example 4. Use of a control potassium channel that does not show constitutive activity: Utility of IK, a calcium-activated potassium channel

Five members of the calcium-activated potassium channel family have been identified, namely SK1, SK2, SK3, SK4 (also called IK) and BK. Physiologically, each of these channels is activated (opened) in response to a rise in intracellular calcium concentration. Pharmacological tools have also been developed for this class of ion channels. For example, the scorpion toxin charybdotoxin is a potent IK channel blocker (Jensen et al.,1998), and the compound EBIO is an IK/SK channel opener (Syme et al.,2000).

Figure 6a shows electrophysiological data from a CHO IK potassium channel stable cell line. Under recording conditions where the free calcium concentration in the patch pipette is set at 100nM, little or no potassium current was recorded. However, upon application of an IK channel opener, a concentration-dependent activation of an inwardly rectifying potassium current was seen (Figure 6a). Application of the IK channel opener also had a marked effect upon the resting membrane potential. As shown in Figure 6b, in the absence of compound the resting potential was very depolarized at approximately –15mV (which approximates to the 'native' resting membrane potential in this batch of CHO

cells). Addition of the IK opener led to a concentration-dependent hyperpolarisation of the cell, with maximal concentrations re-setting the resting potential to –83mV. Under the recording conditions used in this experiment the predicted potassium equilibrium potential is –85mV. Thus, by activating the IK potassium channel it has been possible to hyperpolarise the cell's resting potential from –15mV to the potassium equilibrium potential.

Figure 7 shows data from a FLIPR/DiBAC assay using the same IK channel opener. As seen with the electrophysiology recordings, a concentration-dependent hyperpolarisation (decrease in DiBAC counts) was seen suggesting that this method can be used to re-set membrane potential in a plate-based assay. There is an alternative method whereby the IK channel can be used as control channel to regulate cellular resting potential: If IK-expressing cells are pre-incubated in a maximal concentration of the IK opener, then extremely tight control of resting membrane potential can be achieved by varying the extracellular potassium concentration. Further details of this method are described in Example 5.

Example 5. Use of TREK as a control potassium channel to enable the development of a plate-based assay for the R-type calcium channel.

Development of an assay for voltage-gated ion channels, such as voltage-gated sodium channels or calcium channels, can be very difficult as the physiological stimulus for channel opening is a change in membrane potential. In the absence of assay technology that utilizes electrical stimulation (such technology is still in the testing stage), most experimenters will choose to activate voltage-gated channels by applying a step-wise increase in extracellular potassium concentration to the cell, thereby shifting the potassium equilibrium potential and depolarizing the cell accordingly. However, with a number of voltage-gated channels the steady-state inactivation curve lies at rather hyperpolarised potentials. Thus, for the R-type calcium channel (molecular correlates = α1E alpha subunit plus β3 subunit), 50% of channels are inactivated at a resting membrane potential of –75mV. To overcome this problem, and to enable assay development for the R-type channel, we have developed a stable cell line that

expresses calcium channel α1E plus β3 subunits (the target ion channel) as well as the TREK potassium channel (the control ion channel). As shown in Table 1 co-expression with TREK, and the subsequent hyperpolarisation of the resting membrane potential, allowed development of a FLIPR/Fluo-4 plate-based assay for the R-type (α1E) calcium channel.

Properties of different HEK cells

	I_{Ca}	I_{TREK}	RMP (mV)	FLIPR response
HEK 293 human α1E/β3	✓	X	-10	X
HEK 293 human TREK	X	✓	-75	X
HEK 293 α1E/β3 +TREK	✓	✓	-75	✓

Table 1.

As discussed in previous examples, in the TREK/α1E/β3 stable cell line which expresses a constitutive potassium conductance very tight control of membrane potential can be achieved by varying the extracellular potassium concentration. Figure 8 shows the results from a FLIPR experiment where changes in extracellular potassium concentration were used in two ways: First, pre-incubation in buffers of varying potassium concentration was used to set the resting membrane potential of the TREK/α1E/β3 cells. Second, on-line addition of a high potassium buffer solution was used to activate the voltage-gated target ion channel. Through the use of TREK as the control channel, and changes in extracellular potassium to control the resting membrane potential it has been possible to construct a steady-state inactivation curve and an activation curve for

the R-type calcium channel in a plate-based assay format. These data are described in more detail in the following paragraph.

In the experiment shown in Figure 8a, cells were pre-incubated in buffer with various concentrations of extracellular potassium and addition of 60mM potassium was used to activate the R-type calcium channel. Fluo-4 fluorescence transients were recorded in response to on-line addition of 60mM potassium and the amplitude of these responses in plotted against the potassium concentration used during the pre-incubation period. The results from this experiment clearly show that hyperpolarisation of the resting membrane potential through expression of TREK has enabled development of a robust assay for the R-type channel. Just a small increase in extracellular potassium concentration, from 2 to 6mM during the pre-incubation period is sufficient to decrease the amplitude of the target channel response, and increasing potassium concentration to 11mM abolished the Fluo-4 transient.

Figure 8b shows an activation curve for the R-type channel. In this experiment potassium concentration during the pre-incubation period is kept constant at 2.68mM (which will set the membrane potential such that no steady-state inactivation takes place, see left hand panel) and varying amounts of potassium are added to activate the target channel. As a result of the high potassium conductance of the TREK/α1E/β3 stable cell line a graded depolarization is possible resulting in an activation curve for the R-type channel which is comparable to that recorded by electrophysiology.

An identical TREK/α1E/β3 stable cell line can be used to develop a FLIPR/Fluo-4 assay for the identification of TREK blockers. Pre-incubation with a test compound that blocks TREK will collapse the membrane potential, thereby depolarizing the TREK/α1E cells and inactivating the R-type calcium channels. This will result in a loss of the Fluo-4 transient seen upon on-line application of 60mM potassium.

An R-type calcium channel assay can also be configured using transient expression of TREK into a stable cell line expressing the α1E and β3 calcium channel sub-units. Figure 8C shows data from an FLIPR / Fluo-4 experiment

where BacMam TREK virus was transduced into a HEK293 α1E/β3 stable cell line. In the absence of TREK no response was seen to on-line addition of a high potassium buffer solution (final KCl concentration = 37.5mM). In contrast, robust FLIPR responses were consistently seen in cells transduced with BacMam TREK virus. This experiment was repeated on 3 occasions with similar results (n=48 wells per assay).

Example 6. Use of valinomycin to enable the development of a plate-based assay for the R-type calcium channel.

A potassium-selective ionophore such as valinomycin can be used as an alternative to a control potassium channel. Valinomycin will behave in an identical manner to a constitutively active, non-voltage gated potassium channel such as the two-pore channel (TRAAK, TASK-3, TREK etc.; see Example 2), namely it will insert a steady-state potassium conductance into the plasma membrane and drag the membrane potential towards the potassium equilibrium potential (E_K). In the presence of valinomycin, the membrane potential is approximately equal to E_K, and therefore it is possible to set the required cellular membrane potential simply by adjusting the extracellular potassium concentration.

An alternative way of screening a cell line expressing the R-type voltage-gated calcium channel is to hyperpolarise the resting membrane potential using the potassium ionophore valinomycin rather than through the use of TREK as a control potassium channel. In these experiments cells are pre-incubated with valinomycin and resting membrane potential is set in an identical manner to that described in Example 5, i.e. through changes in the potassium concentration of the buffer solution used for the pre-incubation period. Figure 9 shows FLIPR/Fluo-4 data generated in a HEK293 α1E/β3 calcium channel cell line that had been pre-incubated in low potassium buffer plus various concentrations of valinomycin. Pre-incubation with valinomycin restored a calcium channel response to addition of 60mM potassium, with the amplitude of the Fluo-4 transient increasing with increasing concentrations of valinomycin.

Example 7. Use of valinomycin to enable development of a plate-based assay for the detection of use-dependent blockers of the R-type calcium channel.

Re-setting the resting membrane potential of the HEK293 α1E/β3 cell line with valinomycin has enabled us to develop an assay for use-dependent antagonists of the R-type calcium channel. To measure use-dependence, concentration-response curves are constructed for the test compound using two different assay methodologies: In the first protocol, HEK293 α1E/β3 cells are pre-incubated with valinomycin plus test compound, and then challenged with a high potassium solution to sample the number of R-type channels available for opening (essentially, an identical assay to that described in Example 6). In the second protocol two depolarising potassium stimuli are applied: an initial high potassium stimulus to 'gate' the calcium channels in the presence of test compound, followed by a wash step and then finally a second high potassium stimulus to sample the number of R-type channels available for opening. Use-dependent compounds would be expected to show an increased potency in protocol 2, since the open and inactivated states of the R-type channel are exposed to test compound during the first high potassium stimulus.

Figure 10a shows the results of a use-dependence assay for the R-type calcium channel. With the test compound shown, a clear left-shift in IC_{50} was recorded when a pre-activation with high potassium solution was added to the experimental protocol. The use-dependence of this compound was confirmed by electrophysiology. Figure 10b shows mean data from patch clamp experiments with the same HEK293 α1E/β3 cells that were used in the FLIPR/Fluo-4 assay. When test compound was applied to the cell during a continuous voltage-clamp pulse protocol, a marked inhibition of the calcium channel current was seen. In contrast, application of test compound in the absence of voltage-clamp pulses had little effect upon calcium current amplitude (illustrated by the small difference in current amplitude between the final 'control' voltage-clamp pulse and the first pulse upon re-starting the pulse protocol) suggesting that gating of the calcium channel is required for binding of the channel blocker.

Example 8. Development of a voltage-dependence assay using the TREK/α1E/β3 stable cell line (theoretical example)

In the examples detailed above we have shown that the membrane potential of a TREK/α1E/β3 stable cell line can be manipulated in a predictable manner simply by altering the extracellular potassium concentration. It should therefore be possible to set up cells with two different membrane potentials, and by comparing the potency of an R-type calcium channel blocker across the two groups of cells we should be able to configure a plate-based assay to detect voltage-dependence. A theoretical method for such an experiment is outlined below:

1) In a pilot experiment, construct an inactivation curve for the R-type calcium channel (the target channel) by pre-incubating cells in varying concentrations of extracellular potassium, and then gating the calcium channel with on-line addition of 60mM potassium solution (identical experiment to that described in Example 5 and shown in Figure 8a).
2) From the results of this experiment two potassium concentrations will be selected such that a robust calcium channel signal (response to 60mM K addition) is seen at each concentration, and that the range of potassium concentrations (and therefore membrane potentials) during the pre-incubation period is as large as possible.
3) Cell plates will then be set up for the TREK/α1E/β3 stable cell line & cells loaded with Fluo-4 calcium indicator.
4) For the pre-incubation period, the cell plates will be split into two sections – half the plate will be pre-incubated in low potassium buffer (to give a hyperpolarised membrane potential) and the other half of the plate in a higher potassium buffer (to give a more depolarized membrane potential).
5) Test compound will also be added to the pre-incubation buffer, at a range of concentrations, to allow construction of a concentration response curve.
6) The FLIPR assay will then be carried out , with on-line addition of 60mM potassium to each well to activate the R-type calcium channels and evoke a Fluo-4 calcium response.

7) Concentration-response curves will be constructed for the test compound at each pre-incubation potassium concentration. While a clear decrease in signal amplitude will be seen in the cells pre-incubated in high potassium solution, comparison of normalized concentration-response curves should reveal whether or not the test compound is voltage-dependent.
8) A voltage-dependent blocker of the R-type calcium channel would be expected to show a higher potency in cells pre-incubated in high potassium buffer, which sit at a relatively depolarized membrane potential.

Example 9. Use of IK as a control ion channel to modulate the kinetics and amplitude of a voltage-gated sodium channel response.

In addition to its use in developing a plate-based assay for a problematic ion channels (e.g. R-type calcium channel) and in developing a voltage-dependence assay, a control channel can also be used to improve the signal-to-noise of a plate-based assay. An example of this utility is shown in Figure 11. CHO cells express a small endogenous TTX-sensitive sodium current, and we have shown that activation of these sodium channels with scorpion toxin leads to a depolarization which can be detected in a FLIPR/DiBAC assay (see control in Fig.11). In a CHO IK stable cell line, application of an IK channel opener leads to hyperpolarisation of the resting membrane potential (see Fig.6b,7) and to a shift in the response to scorpion toxin. Thus, in the presence of IK opener the response to scorpion toxin is larger, and more rapid than that seen in control experiments in the same cell line. This may be due to a decrease in the number of inactivated sodium channels (& therefore a greater number of channels available for opening), to an increase in driving force for sodium flux, or to a combination of the two factors.

Example 10. Use of TREK as a control channel to reverse the cytotoxicity associated with functional expression of the NMDA receptor.

Functional expression of recombinant NMDA receptors in mammalian cells has been linked to cell death (Anegawa et al., 1995), thereby compromising attempts

to develop cell-based assays for this channel class. Several methods have been employed in attempts to overcome this problem, including the use of inducible expression systems and pre-incubation with the antagonist ketamine (Priestley et al., 1995).

A unique feature of the NMDA receptor is dual dependence of function on agonist binding and membrane potential. The latter is mediated by voltage-dependent block of the channel by sub-millimolar concentrations of extracellular magnesium (Dingledine et al., 1999). Thus, for receptors containing NR2A or NR2B, Mg^{2+} binds with a K_d of ~10μM at –80mV whereas potency at 0mV is much lower (K_d ~5mM) (Wollmuth et al., 1998). Figure 12 shows data from an experiment in which we have taken advantage of this property of the NMDA receptor to develop an assay in which co-expression of the TREK potassium channel protects NMDA receptor-expressing cells from cytotoxicity.

As described in previous examples, expression of the TREK potassium channel in HEK293 cells re-sets the resting membrane potential to a value close to the potassium equilibrium potential. At this membrane potential, one would expect the NMDA channel to be blocked by extracellular magnesium thereby protecting the cells from NMDA-receptor mediated cytotoxicity.

Figure 12 shows the results of an experiment where TREK was used to protect HEK293 cells from NMDA-induced cytotoxicity. NMDA receptor sub-units (NR1 + NR2A) were transiently transfected into wild type HEK293 cells and TREK HEK293 cells (stable cell line) and cells were grown for 48hrs in the presence of the co-agonists glutamate and glycine. Cell survival was assayed using a trypan blue exclusion assay. All data are normalised to the results obtained in control experiments using the empty vector pCDNA3.1V5 hisTOPO.

In wild type HEK cells, cell survival at 48hrs post NMDA receptor transfection was 55.1 ± 2.4% (normalised to vector control, n=7). In TREK HEK293 cells, cell survival after NMDA transfection was 84.4 ± 2.5% (n=7). These data are significantly different (T-test (un-paired), $P<0.01$, n=7).

Methods

Each of the experiments described in this patent were carried out using one of the following techniques: two-microelectrode voltage-clamp in Xenopus oocytes, whole-cell patch clamp in mammalian stable cell lines, membrane potential recordings in Xenopus oocytes or mammalian cell lines, FLIPR/Fluo-4 plate-based assay or FLIPR/DiBAC plate-based assay. General methods for each of these techniques are described below and further details are given where appropriate in the figure legends and text accompanying the individual examples.

Two microelectrode voltage-clamp in Xenopus oocytes

Adult female *Xenopus laevis* (Blades Biologicals) were anaesthetised using 0.2% tricaine (3-aminobenzoic acid ethyl ester), killed and the ovaries rapidly removed. Oocytes were de-folliculated by collagenase digestion (Sigma type I, 1.5 mg ml^{-1}) in divalent cation-free OR2 solution (in mM): 82.5 NaCl, 2.5 KCl, 1.2 NaH_2PO_4, 5 HEPES (4-(2-hydroxethyl)-1-piperazine ethanesulphonic acid); pH 7.5 at 25°C. Single stage V and VI oocytes were transferred to ND96 solution (in mM): 96 NaCl, 2 KCl, 1 $MgCl_2$, 1.8 $CaCl_2$, 5 HEPES; pH 7.5 at 25°C, which contained 50$\mu g ml^{-1}$ gentamycin, and stored at 18°C.

KCNQ2 and KCNQ3 (both in pCIH6) plasmid DNA was linearised and RNA transcribed using SP6 polymerase (mMessage machine, Ambion). Equimolar KCNQ2 and KCNQ3 m'G(5')pp(5')GTP capped cRNA was injected into oocytes (20-50ng per oocyte) and whole-cell currents were recorded using two-microelectrode voltage-clamp (Geneclamp amplifier, Axon instruments Inc.) 3 to 5 days post-RNA injection. Microelectrodes had a resistance of 0.5 to 2MΩ when filled with 3M KCl. In all experiments oocytes were voltage-clamped at a holding potential of −100mV in ND96 solution (superfused at 2ml per min.) and retigabine was applied by addition to this extracellular solution. Retigabine (D-23129; N-(2-amino-4-(4-flurobenzylamino)-phenyl) carbamic acid ethyl ester or 2-amino-4-(4-fluorobenzylamino)-1-ethoxycarbonylaminobenzene) as its

dihydrochloride salt, was prepared in a four stage process from 2-nitro-4-aminoaniline and 4-fluorobenzaldehyde according to the method outlined in US patent 5,384,330 (Dieter et al.(Asta Medica),1995). A 10mM retigabine stock solution was made up in water prior to each experiment. Voltage-protocols were generated using pCLAMP8 software (Axon Instruments) and a P/N leak subtraction protocol was used throughout

In a number of experiments the effects of retigabine on oocyte membrane potential were studied by impaling the oocyte with a single microelectrode, and measuring membrane potential using the Geneclamp amplifier.

Whole-cell patch clamp

Whole-cell patch clamp recordings were made using standard methods (Dupere et al.,1999). Briefly, cells were grown on a glass coverslip, placed into a recording chamber (0.5ml volume) and superfused with an extracellular recording solution at 2 ml min^{-1}. The extracellular recording solution contained (in mM): 130 NaCl, 5 KCl, 15 HEPES, 2 $CaCl_2$, 1 $MgCl_2$, 30 glucose; pH 7.3 with NaOH. Patch electrodes had resistances of 2 to 6MΩ when filled and the pipette-filling solution contained (in mM): 140 KCl, 10 HEPES, 4 $MgCl_2$, 10 EGTA (ethylene glycol-bis(β-aminoethyl ester) N,N,N',N-tetra acetic acid, K salt); pH7.3 with KOH. Currents were recorded at room temperature using an Axopatch 200B amplifier (Axon Instruments Inc.), and test compounds were applied through addition to the superfusate. Acquisition and analysis utilised pCLAMP software. Currents were digitised at 10 kHz and filtered at 5 kHz before being stored on computer for later analysis.

In a number of experiments membrane potential recordings were made from mammalian cells, either using the whole-cell patch clamp configuration described above, or the amphotericin perforated patch-clamp technique. In each case, membrane potential was monitored by switching the patch clamp amplifier to the 'I=0' voltage-follower mode.

FLIPR assays

In a number of experiments, fluorescence-based measurement of membrane potential or intracellular calcium were carried out using the FLIPR (Fluorometric Imaging Plate Reader; Molecular Devices) in conjunction with either the potentiometric, fluorescent dye $DiBAC(4)_3$ or the calcium-sensitive dye Fluo-4. Full background information and standard methods for FLIPR/DiBAC and FLIPR/Fluo-4 assays are detailed in literature provided by Molecular Devices. Details of experimental protocols used for the examples detailed in the patent are given below:

FLIPR/DiBAC experiments

CHO hKCNQ 2/3 and CHO hIK cells were seeded into 96 well black, clear-bottom plates at 45,000 cells per well. FLIPR assays were carried out 2 days post-seeding. $DiBAC(4)_3$ was prepared as a 50mM stock solution in DMSO. All DiBAC containing solutions were made up in glass containers, and plasticware (tips, reservoirs, compounds plates) were pre-soaked with 10μM DiBAC to block absorption. Cell media was removed and cells were washed once with 180μl DiBAC buffer (in mM: 145 NaCl ; 5 KCl; 2 $CaCl_2$; 0.8 $MgCl_2$; 10 HEPES; 10 Glucose; pH 7.4 plus 5μM DIBAC) and then incubated in the same buffer solution for 45 min at 37°C. In experiments where pre-incubation with an ion channel opener/blocker was required, compound was added after the 45min incubation and then the plate was incubated for another 20 min. At the end of the pre-incubation period, plates were placed on the FLIPR and compound were added by on-line addition as required. Scorpion toxin was used at a final concentration of 40μg per ml.

FLIPR/Fluo-4 experiments

TREK/α1E/β3 HEK293 experiments

Cells were plated in 384-well, poly-D-lysine coated plates 24hrs before use at a seeding density of 5,000 cells (25μl media) per well. Experiments were carried out using a Tyrode buffer solution that contained (in mM): 145 NaCl; 2.5 KCl; 10 HEPES; 10 glucose; 1.2 $MgCl_2$;1.5 $CaCl_2$; pH 7.4 @ room temperature. In experiments where potassium concentration in the pre-incubation buffer was set at various levels between 2mM and 145mM, KCl was substituted for NaCl in the Tyrode's solution. At the start of the experiment, cells were loaded with cell media containing 3μM probenicid and 2.25μM Fluo-4 (addition volume = 15μl per

well) for 1h at 37°C. Cells were then washed three times with Tyrode's buffer (at the required pre-incubation potassium concentration), leaving a final buffer volume of 40µl per well. For the theoretical experiment detailed in Example 8, 10µl of antagonist/test compound (in Tyrode's buffer) would be added to the cells at this stage, and the cell plates incubated for a further 20mins @ 37°C. The cell plate was then placed on the FLIPR and an on-line addition of 10µl high potassium buffer was applied to the cells to activate the α1E channel. For calcium channel activation curves, cells were stimulated with KCl (in Tyrode's buffer) at final well concentrations of between 0.3 and 100mM (11 data points at ¼ log dilution).

TREK BacMam / α1E/β3 HEK293 experiments

α1E/β3 HEK293 cells were transduced with TREK bacculovirus through addition of 5% virus (by volume) to the tissue culture media. Following 3 days exposure to TREK virus, cells were plated in 384-well, poly-D-lysine coated plates at a seeding density of 10,000 cells (50µl media) per well. Experiments were carried out 24h post-plating using a buffer solution that contained (in mM): 145 NaCl; 5 KCl; 0.35 KH_2PO_4; 0.3 NaH_2PO_4; 0.8 $MgSO_4$; 1 $CaCl_2$; 10 HEPES; 5.6 glucose; pH 7.4 @ room temperature. At the start of the experiment, cells were loaded with cell media containing 250µM sulphinpyrazone and 1.092µM Fluo-4 (addition volume = 35µl per well) for 1h at 37°C. Cells were then washed twice with buffer leaving a final buffer volume of 60µl per well. The cell plate was then placed on the FLIPR and an on-line addition of 20µl high potassium (150mM) buffer was applied to the cells to activate the α1E channel (final potassium concentration = 37.5mM).

Valinomycin / α1E/β3 HEK293 experiments

Valinomycin experiments using the α1E/β3 HEK293 cell line were carried out in 96 well FLIPR. As with the TREK/ α 1E/ β3 HEK293 cell line, experiments were carried out using a Tyrode buffer solution that contained (in mM): 145 NaCl; 2.5 KCl; 10 HEPES; 10 glucose; 1.2 $MgCl_2$;1.5 $CaCl_2$; pH 7.4 @ room temperature. In experiments where the potassium concentration in the pre-incubation buffer was varied, KCl was substituted for NaCl. α1E/ β3 HEK293 cells were plated in 96-well, poly-D-lysine coated plates 36hrs before use at a seeding density of

30,000 cells (100µl media) per well. At the start of the experiment, cells were loaded with cell media containing 6µM probenicid and 2.4µM Fluo-4 (addition volume = 25µl per well) for 1h at 37°C. A solution containing 25µl test compound plus 10µM valinomycin was then added to cells and incubated for 10mins @ 37°C. For the KCl pre-activation, cells were stimulated with 50µl KCl (31.6mM per well) for 5min. Cells were then washed three times with Tyrode's buffer, leaving a final buffer volume of 125µl per well. 25µl test compound was then re-applied to cells and the cell plate was placed on the FLIPR. On-line addition of 50µl KCl was used to activate the α1E channel.

Baculovirus (BacMam) expression of Kir6.2 / SUR1.

CHO cells were maintained as spinner culture in Excel 301 media supplemented with 1X Pen/Strep and 5% FBS. Cells were split 1:5-1:10 on a MWF schedule. CHO cells were plated at 20,000 cells per well in 96 well black/clear bottom FLIPR plates using DMEM/F12 supplemented with 1X Pen/Strep and 5%FBS (CHO Media). After the cells were allowed to attach for 2 hours, the media was removed and 50µl of virus diluted in CHO media to the indicated concentration was added. K_{ATP} activity was measured 18-24 hours later in a FLIPR/DiBAC assay.

Use of TREK to develop a cell protection assay.

Untransfected HEK293 cells and HEK293 cells stably expressing the TREK potassium channel were plated out in 24 well dishes at a seeding density of 1 x 10^5 cells per well. HEK293 cells were cultured in DMEM Hams F12 mixture, 10% fetal bovine serum, 2mM L glutamine and 1% non essential amino acids. For the recombinant TREK cell line this media also included 500µg/ml of the antibiotic G418. The cells were grown in a 5% CO_2 incubator at 37°C. At a time-point of 24h post-plating the cells were transfected with cDNAs encoding the NMDA receptor subunits NR1 (0.2µg) and NR2A (0.8µg) in the expression plasmid pCDNA3.1V5 hisTOPO (Invitrogen). Total cDNA in these transfections was therefore 1µg and in control experiments untransfected HEK293 and TREK HEK293 cells were transfected with 1µg of the 'empty' expression plasmid pCDNA3.1V5 hisTOPO per well.

After 6 hours the transfection media was removed and replaced with the normal cell culture media for HEK293 and TREK HEK293 cells respectively. At this point 100μM glutamate and 10μM glycine, the co agonists required for activation of the NMDA receptor, were also included in the cell culture media.

After 48 hours, media was removed and the cells were taken off the plate in 100μl of PBS. 100μl of 0.4% trypan blue (Sigma) was added to each sample and live cells (those cells excluding trypan blue) were counted using a haemocytometer. Cell survival was calculated by dividing the number of live cells expressing NMDA receptors by the number of live cell in the control sample. Each transfection was performed in triplicate.

References

Anegawa NJ, Lynch DR, Verdoorn TA & Pritchett DB (1995) Transfection of N-Methyl-D-Aspartate receptors in a non-neuronal cell line leads to cell death. *J.Neurochem.* **64**:2004-2012.

Bean, BP (1984) Nitrendipine block of cardiac calcium channels: High-affinity binding to the inactivated state. *Proc.Natl.Acad.Sci.* **81**:6388-6392.

Dingledine R, Borges K, Bowie D & Traynelis SF (1999) The glutamate receptor ion channels. *Pharmacol.Rev.* **51(1)**:7-61.

England PJ. (1999) Discovering ion-channel modulators - making the electrophysiologist's life more interesting. *Drug Discov Today* **4**:391-392

Hille B. (1992) Ionic channels of excitable membranes, Second edition. Sinauer Associates Inc.

Jensen BS, Strobaek D, Christophersen P, Jorgensen TD, Hansen C, Silahtaroglu A, Olesen SP & Ahring PK. (1998) Characterization of the cloned human intermediate-conductance Ca2+-activated K+ channel. *Am J Physiol* **275**:C848-56.

Kost TA & Condreay JP. (1999) Recombinant baculoviruses as expression vectors for insect and mammalian cells. *Curr Opin Biotechnol* **10**:428-33

Main MJ, Cryan JE, Dupere JR, Cox B, Clare JJ & Burbidge SA. (2000) Modulation of KCNQ2/3 potassium channels by the novel anticonvulsant retigabine. *Mol. Pharmacol.* **58(2)**:253-62.

Meadows H.J. & Randall A.D. (2001) Functional characterisation of human TASK-3, an acid-sensitive two-pore domain potassium channel. *Neuropharmacology* 40(4):551-559.

Nakahira K, Shi G, Rhodes KJ & Trimmer JS. (1996) Selective interaction of voltage-gated K+ channel beta-subunits with alpha-subunits. *J Biol Chem* **271**:7084-9

Nash MS, Selkirk JV, Gaymer CE, Challiss RA, Nahorski SR.(2001) Enhanced inducible mGlu1alpha receptor expression in Chinese hamster ovary cells. *J Neurochem* **77**:1664-7

Priestley T, Laughton P, Myers J, Le Bourdellés B, Kerby J & Whiting PJ (1995) *Mol.Pharmacol.* **48**:841-848.

Sambrook et al. (1989) Molecular Cloning: a Laboratory Manual. 2nd Edition. CSH Laboratory Press.

Sanguinetti M.C., Curran M.E., Zou A., Shen J., Spector P.S., Atkinson D.L. & Keating M.T. (1996) Coassembly of K(V)LQT1 and minK (IsK) proteins to form cardiac I(Ks) potassium channel. *Nature* **384**:80-3

Schwake M, Pusch M, Kharkovets T & Jentsch TJ. (2000) Surface expression and single channel properties of KCNQ2/KCNQ3, M-type K+ channels involved in epilepsy. *J Biol. Chem.* **275**:13343-8.

Syme CA, Gerlach AC, Singh AK & Devor DC. (2000) Pharmacological activation of cloned intermediate- and small-conductance Ca(2+)-activated K(+) channels. *Am J Physiol* **278**:C570-81

Terstappen GC, Pula G, Carignani C, Chen MX & Roncarati R. (2001) Pharmacological characterisation of the human small conductance calcium-activated potassium channel hSK3 reveals sensitivity to tricyclic antidepressants and antipsychotic phenothiazines. *Neuropharmacology* **40**:772-83

Thomas D, Tovey SC, Collins TJ, Bootman MD, Berridge MJ, Lipp P. (2000) A comparison of fluorescent Ca2+ indicator properties and their use in measuring elementary and global Ca2+ signals. *Cell Calcium* **28**:213-23

Wang HS, Pan Z, Shi W, Brown BS, Wymore RS, Cohen IS, Dixon JE & McKinnon D. (1998) KCNQ2 and KCNQ3 potassium channel subunits: molecular correlates of the M-channel. *Science* **282**:1890-3

Wollmuth LP, Kuner T & Sakmann B (1998) Adjacent asparagines in the NR2 subunit of the NMDA receptor channel control the voltage-dependent block by extracellular Mg^{2+}. *J.Physiol.* **506**:13-32.

Woodhull, A.M. (1973) Ionic blockade of sodium channels in nerve. *J.Gen.Physiol.* **61**:687-708.

Xie X, Lancaster B, Peakman T & Garthwaite J. (1995) Interaction of the antiepileptic drug lamotrigine with recombinant rat brain type IIA Na+ channels and with native Na+ channels in rat hippocampal neurones. *Pflugers Arch* **430**:437-46

Yamakura T. & Shimoji K. (1999) Sub-unit and site-specific pharmacology of the NMDA receptor channel. *Prog.Neurobiol.* **59** 279-298.

Claims

1. A method for identifying an ion channel modulator which exhibits different activity at different membrane potentials comprising:
   a. at a first membrane potential, contacting a test substance with a stable cell line which expresses on its surface a target ion channel to be modulated;
   b. determining the effect of the test substance on the activity and/or expression of the target ion channel, thereby to determine whether the test substance modulates ion channel activity and/or expression;
   c. contacting the test substance with a stable cell line which expresses the target ion channel at a second membrane potential;
   d. determining the effect of the test substance on the activity and/or expression of the target ion channel at the second membrane potential; and
   e. comparing the activity of the test substance on the target ion channel at the two membrane potentials.

2. A method according to claim 1 in which the stable cell line in step c. expresses on its surface the target ion channel to be modulated and a further, control ion channel which can be utilised to control the resting membrane potential of the cell.

3. A method according to claim 1 in which the stable cell line in step c. is the same cell line as used in step a. and expresses on its surface both the target ion channel to be modulated and a further, control ion channel which can be utilised to control the resting membrane potential of the cell.

4. A method according to any one of claims 1 to 3 in which step a. and step c are carried out sequentially.

5. A method according to any one of claims 1 to 3 in which steps a. and c. are carried out simultaneously.

6. A method according to claim 5 in which steps a. and c. are carried out in separate wells of a multi-well microtitre assay plate.

7. A method according to any preceding claim further comprising contacting the test substance with the stable cell line at one or more further membrane potentials, determining the effect of the test substance on the activity and/or expression of the target ion channel and comparing the activity of the substance at the first, second and further membrane potentials.

8. A method according any one of claims 2 to 7 in which the membrane potential is controlled by addition to the stable cell line of an ion channel modulator compound to modulate the activity of the control ion channel.

9. A method according any one of claims 2 to 7 in which the membrane potential is controlled by altering the level of expression of the control ion channel.

10. A method according to claim 8 wherein the ion channel modulator is a channel opener.

11. A method according to claim 8 wherein the ion channel modulator is a channel blocker.

12. A method according to claim 8 wherein the ion channel modulator is a solution of the permeating ion for the control ion channel.

13. A method according to claim 9 in which the level of expression of the control ion channel is adjusted using a BacMam expression system.

14. A method according to any preceding claim in which the control ion channel is a potassium ion channel.

15. A method according to any preceding claim in which the control ion channel is an ionophore.

16. A method according to claim 14 in which the potassium ion channel is a KCNQ channel, an IK potassium channel, a TREK potassium channel or an ATP-sensitive potassium channel.

17. A method according to any preceding claim in which the control ion channel is utilised to hyperpolarise the membrane potential of the stable cell line.

18. A method according to claim 17 in which the hyperpolarisation is such that any detrimental effects caused by the ions that enter the cell via the target ion channel are ameliorated.

19. A method according to claim 17 in which the hyperpolarisation is such that the driving force for the ions that enter the cell via the target ion channel is increased.

20. A method according to claim 17 in which the hyperpolarisation is such that the majority of the target ion channels lie in a non-inactivated state.

21. Use of a stable cell line which expresses on its surface both a target ion channel to be modulated and a further, control ion channel which can be utilised to control the resting membrane potential of the cell, to increase the signal to noise ratio in an assay for modulators of the target ion channel.

22. Use of a stable cell line which expresses on its surface both a target ion channel to be modulated and a further, control ion channel which can be utilised to control the resting membrane potential of the cell, in an assay for the identification of voltage-dependence in candidate ion channel modulators.

23. A method of improving a plate based cellular assay for ion channel activation characterised in that the cellular membrane potential is set to a pre-determined level.

24. A method according to claim 23 further characterised in that the membrane potential is reset to a different level, the test substance re-assayed and the results of the two assays compared.

25. Use of a stable cell line which expresses on its surface both a target ion channel to be modulated and a further, control ion channel which can be

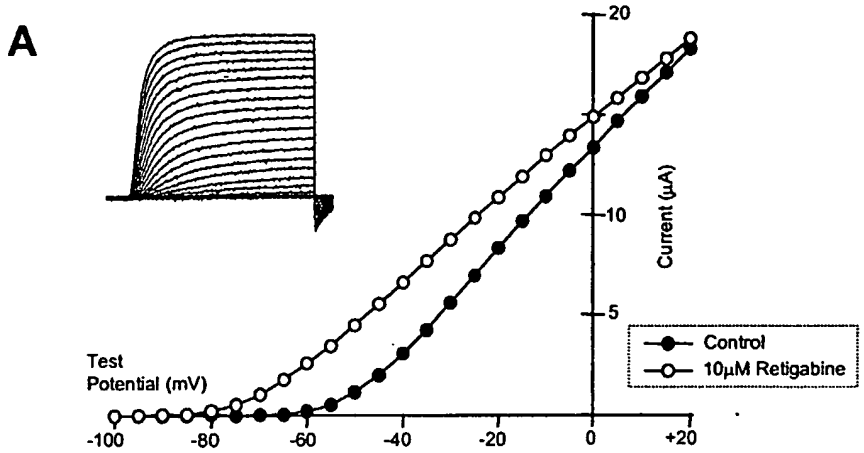
utilised to control the resting membrane potential of the cell, in an assay for the identification of ion channel modulators which exhibit use-dependent block.

26. Use of a stable cell line which expresses on its surface both a target ion channel to be modulated and a further, control ion channel which can be utilised to control the resting membrane potential of the cell, in an assay for the identification of ion channel modulators which exhibit frequency-dependent block.

27. A high throughput method of screening for voltage dependent compounds comprising the steps of contacting a ligand with its respective ion channel in the presence or absence of a test compound and comparing the activation of said channel in the presence or absence of said compound, said method characterised in that the membrane potential at which the assay is conducted has been set to a pre-determined level.

28. A plate based high throughput screen comprising a stable cell line engineered to express a target ion channel of interest, characterised in that the resting membrane potential of said stable cell line has been modulated by the expression of a cloned potassium ion channel.

Figure 1. Voltage-clamp and Membrane Potential recordings from *Xenopus* oocytes expressing the KCNQ2/KCNQ3 potassium channel



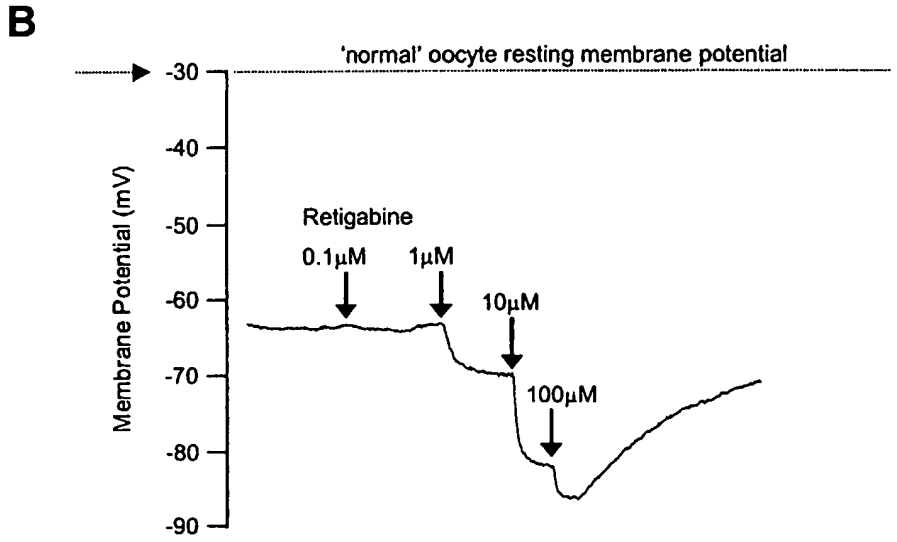
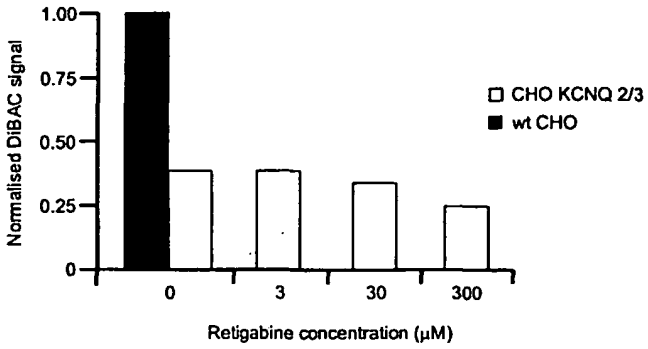


Figure 2. FLIPR DiBAC assay in wild type (wt) CHO cells and a CHO KCNQ2/KCNQ3 potassium channel stable cell line: effects of retigabine and XE991

A



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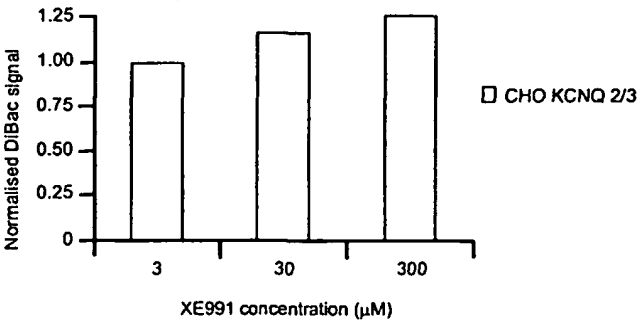


Figure 3. Voltage-clamp recordings from HEK293 cells transiently transfected with the hTRAAK and hTASK-3 two-pore potassium channels

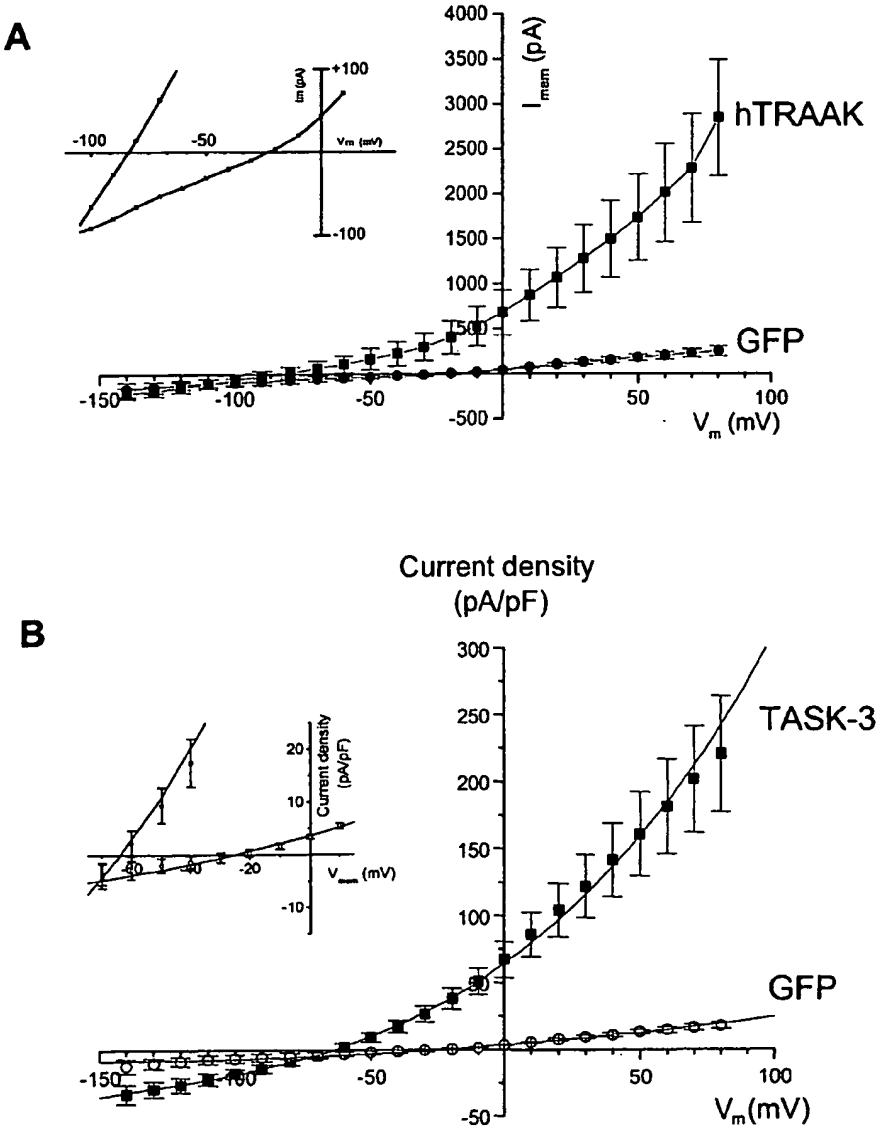


Figure 4. Modulation of TREK and TASK-3 activity through use of a pharmacological blocker and a change in extracellular pH respectively

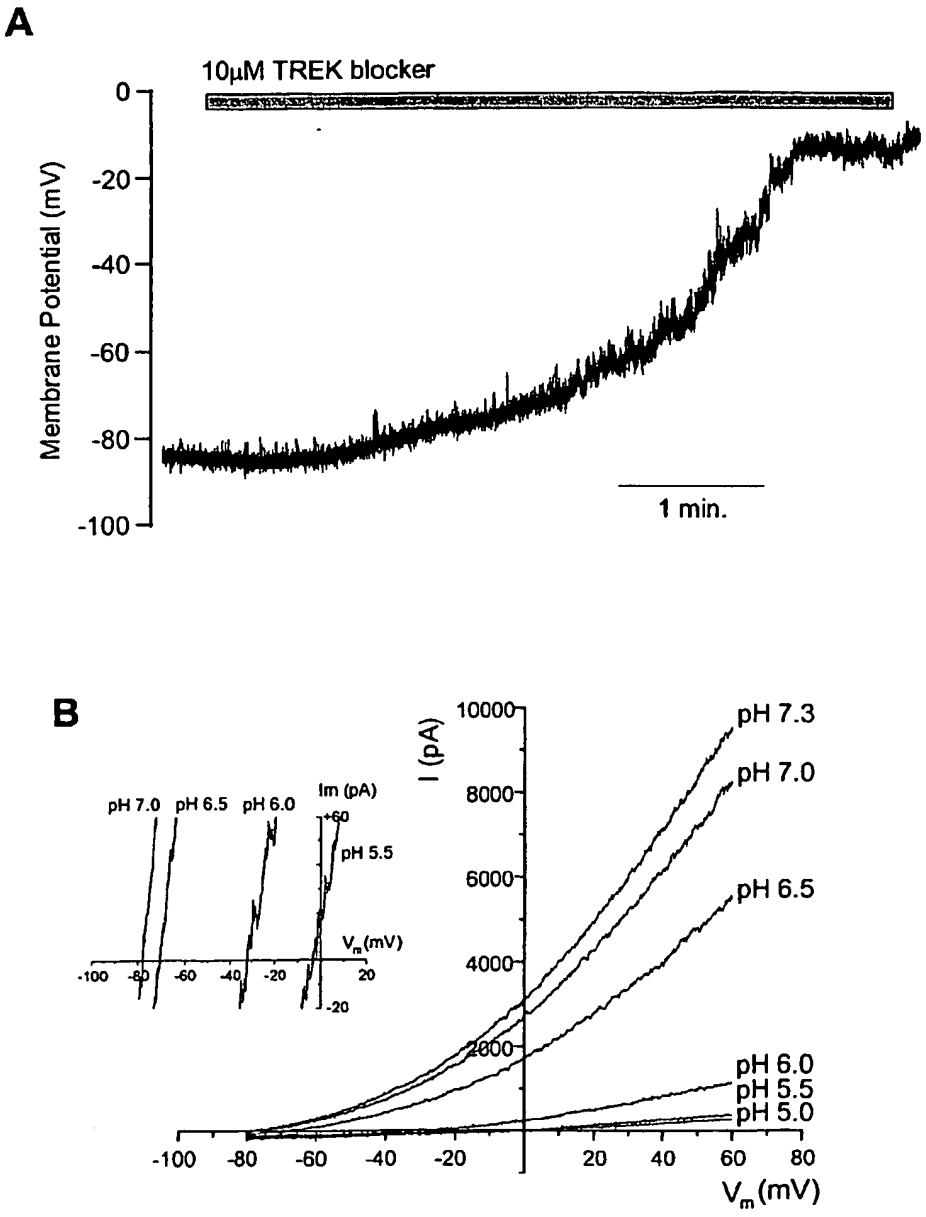


Figure 5. FLIPR/DiBAC experiment showing titration of resting membrane potential using baculoviral expression of Kir6.2 plus SUR1 K_{ATP} sub-units

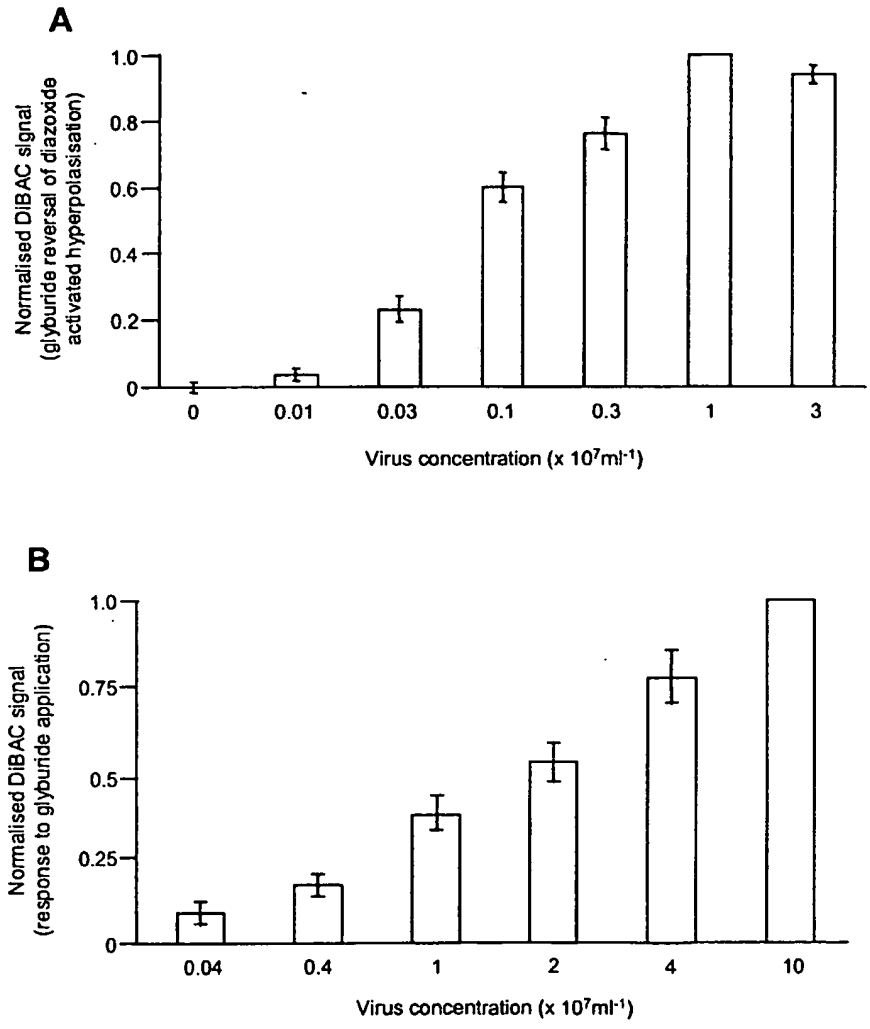
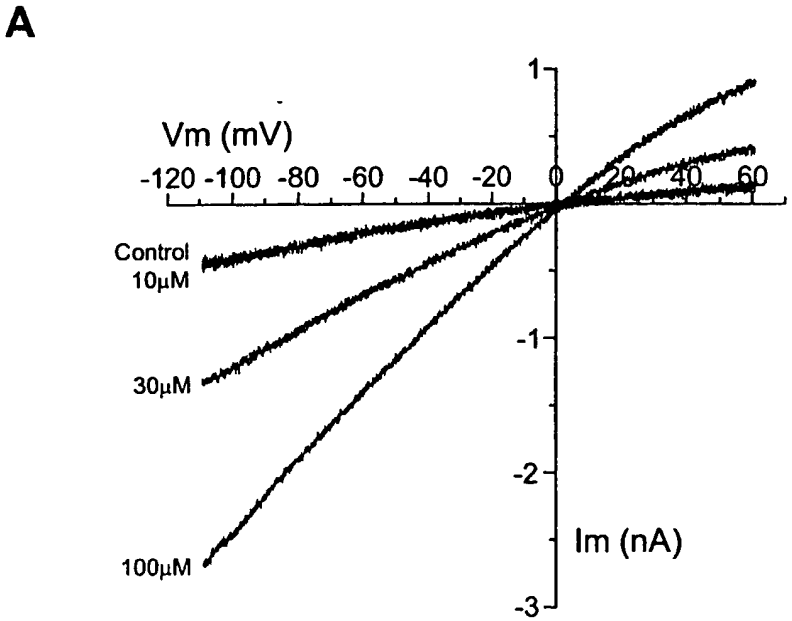


Figure 6. Voltage-clamp and current-clamp recordings from a CHO IK stable cell line: effects of an IK opener



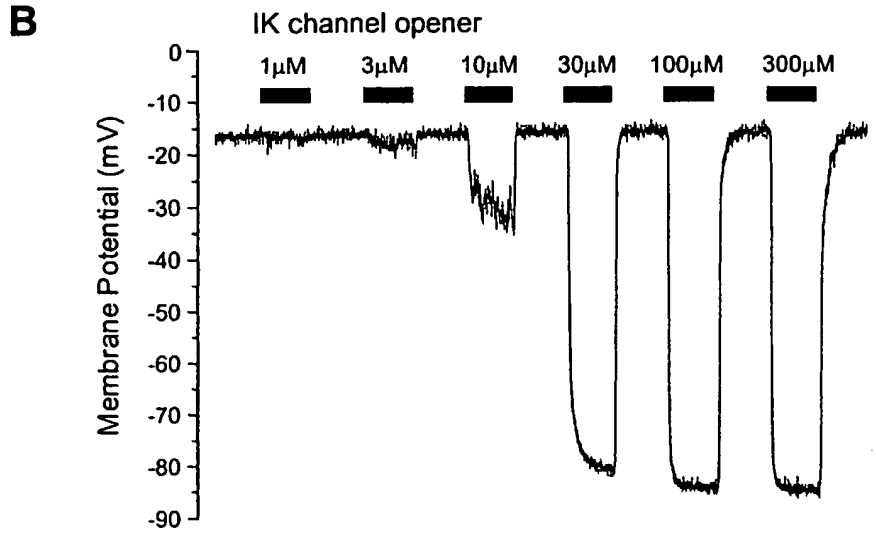


Figure 7. FLIPR/DiBAC experiment showing hyperpolarisation of resting membrane potential in a CHO IK stable cell line following application of an IK opener

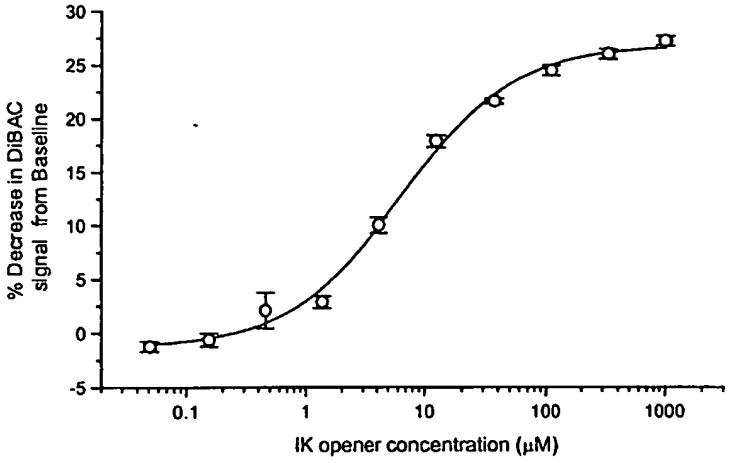


Figure 8. FLIPR/Fluo-4 experiment showing activation and inactivation curves for the R-type calcium channel constructed in a TREK/α1E/β3 cell line

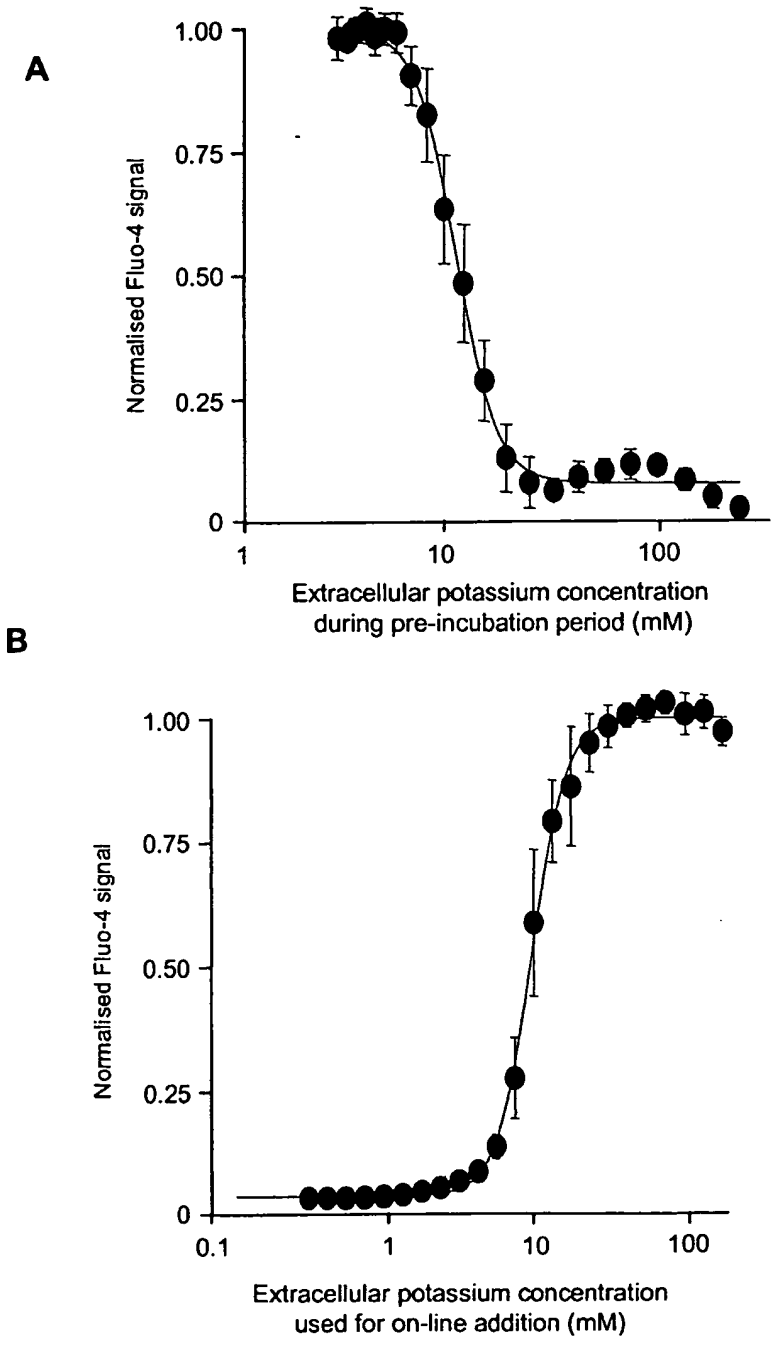


Fig.8C

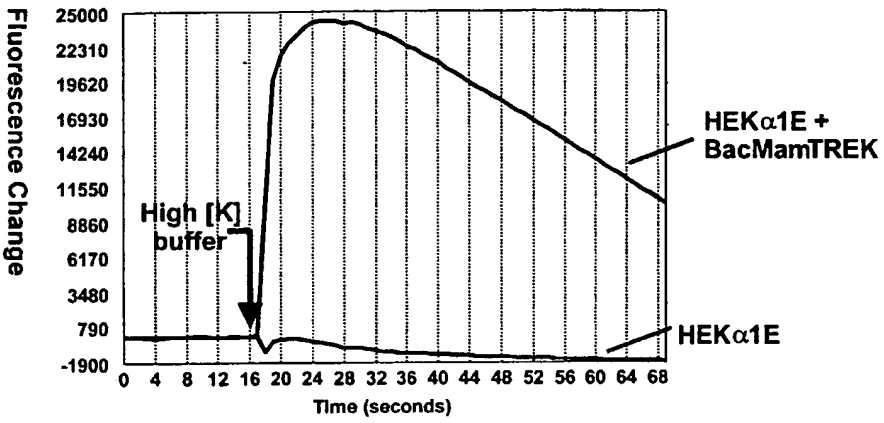

Fluorescence Change
25000
22310
19620
16930
14240
11550
8860
6170
3480
790
-1900
0 4 8 12 16 20 24 28 32 36 40 44 48 52 56 60 64 68
Time (seconds)
High [K] buffer
HEKα1E + BacMamTREK
HEKα1E

Figure 9. FLIPR/Fluo-4 experiment illustrating the use of valinomycin as an alternative to a control potassium channel to enable a plate-based assay for the R-type calcium channel

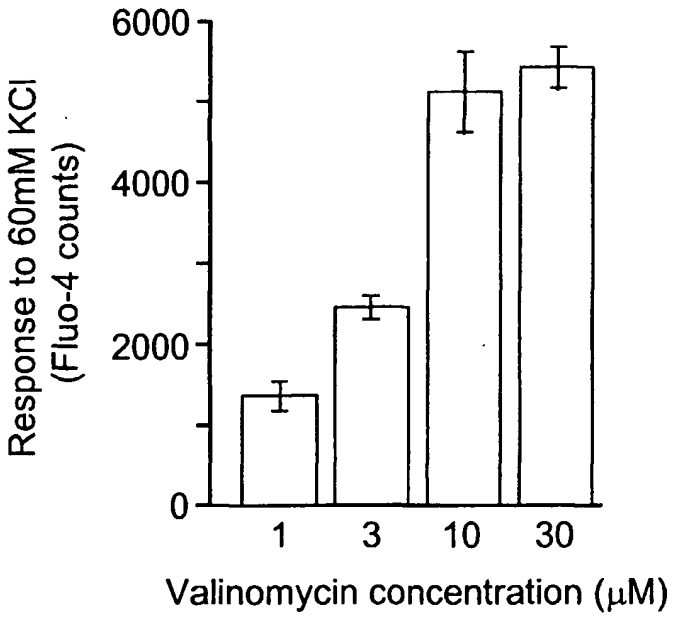
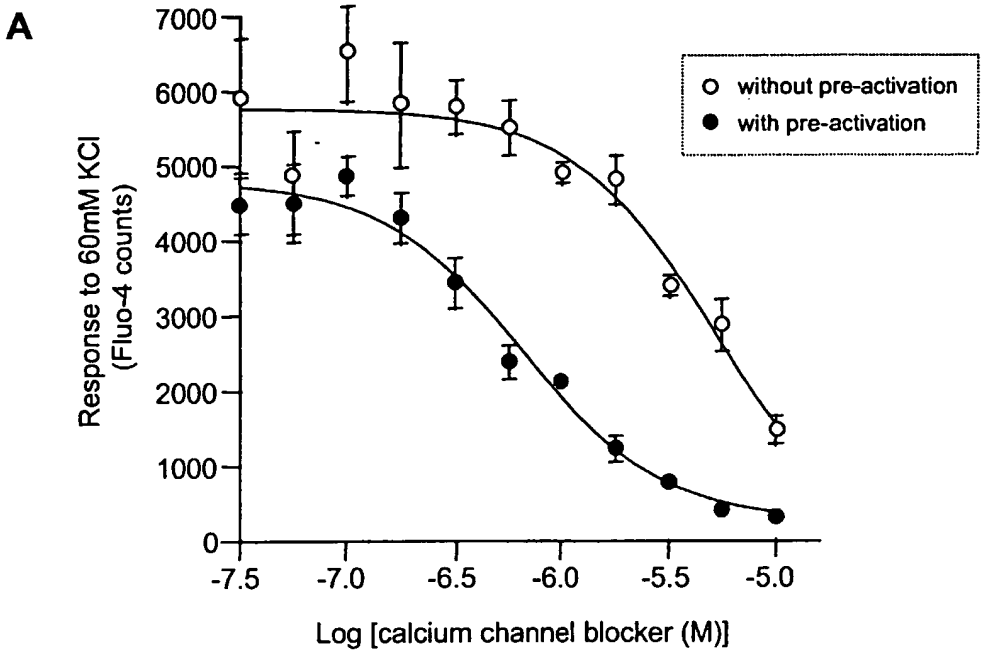


Figure 10. Identification of a use-dependent calcium channel blocker in FLIPR/Fluo-4 and electrophysiology assay formats



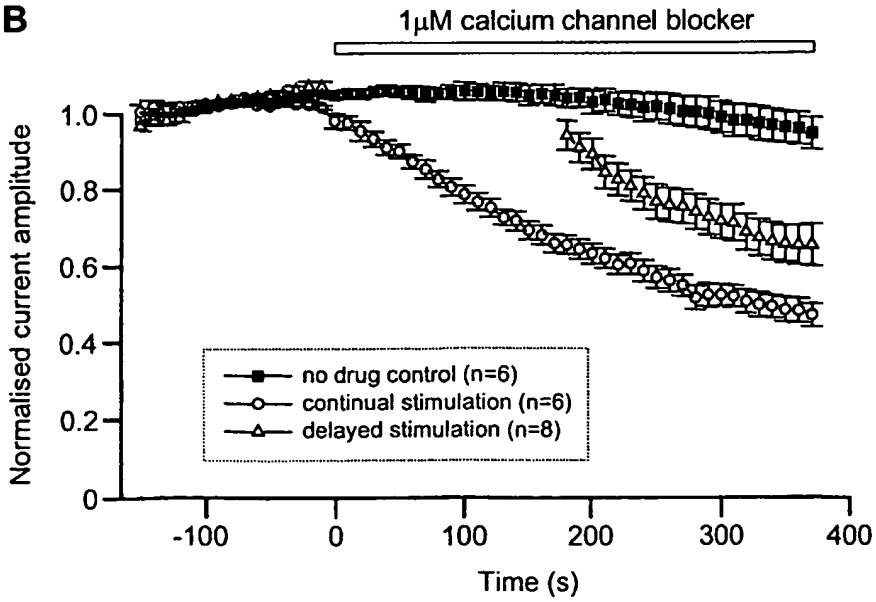


Figure 11. Modulation of the response to Scorpion toxin in a CHO IK stable cell line by pre-incubation in the presence of an IK channel opener.

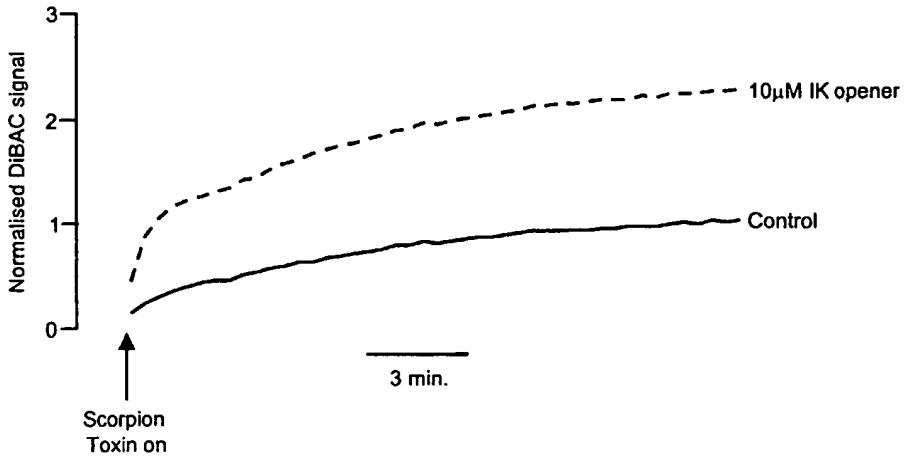


Figure 12. Co-expression of the TREK potassium channel to protect HEK293 cells from NMDA receptor induced cytotoxicity